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TUMOR NECROSIS FACTOR ALPHA PRODUCTION IN A GUINEA-PIG MODEL OF ASTHMA

by

DEBORAH EILEEN BROWN ©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF PHARMACY & PHARMACEUTICAL SCIENCES

EDMONTON, ALBERTA

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ABSTRACT

Tumor necrosis factor alpha (TNFα) is likely involved in the pathogenesis of inflammatory conditions such as asthma (reviewed in Chapter 1). We first hypothesized that TNFα was an early mediator released into the bronchoalveolar lavage (BAL) fluid after antigen challenge of passively immunized guinea-pigs, and that the alveolar macrophage (AM) was a source of enhanced TNFα production. We determined whether tachykinin neuropeptides partly regulated this process. *In vivo* antigen challenge resulted in early production of TNFα into the BAL fluid. Further, AM collected from the BAL were upregulated to produce TNFα and interleukin-6, and neuropeptides indirectly regulated this process. Next we hypothesized that thalidomide inhibits LPS-induced TNFα production from guinea-pig AM, *in vitro,* and demonstrated that the (-) enantiomer was more potent than the (+) enantiomer in this effect. We then tested the effectiveness of thalidomide at reducing airway hyperreactivity, *in vivo.* Thalidomide reduced airways' responsiveness to histamine in antigen challenged animals. We then tested the hypothesis that allergen-activation of guinea-pig AM to secrete TNFα occurs through cross-linking of receptors for IgG (FcγR). Cross-linkage of cell-bound IgG on AM by addition of soluble antigen or anti-IgG antibody did not result in enhanced TNFα production. In these studies, the binding affinity for whole IgG was determined to be 1.24×10^{-9} M and the number of binding sites per cell to be 1.54×10^{5}.

In summary, TNF α is produced into the BAL shortly after antigen challenge of passively immunized guinea-pigs and the AM recovered from lavage fluid are upregulated to produce both TNFα and IL-6. Also, neuropeptides indirectly regulate this process. Thalidomide inhibits histamine-induced increases in airway resistance in passively immunized and challenged guinea-pigs, likely through its immune-modulating effects. Also, IgG binds to guinea-pig AM with high affinity, however cross-linking of

FcγR on AM does not result in TNFα production. Thus, the pro-inflammatory cytokine, TNFα, is detectable after antigen challenge in our guinea-pig model of asthma, and is likely involved in the generation of airways' inflammation and airway hyperreactivity that is characteristic of asthma.

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This thesis is dedicated to those I love:

My beautiful children,

Bryce, Nicole, Lyndan and Steven, and

My closest friend and life-long companion,

Michael James.

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LIST OF ABBREVIATIONS AND SYMBOLS

AA	Arachidonic acid
ABTS	2,2'-Azinobis (3-ethylbenzthiazolinesulfonic acid)
Ach	Acetylcholine
ADCC	Antibody-dependent cell-mediated cytotoxicity
AM	Alveolar macrophage
APC	Antigen presenting cell
BAL	Bronchial alveolar lavage
BALF	Bronchial alveolar lavage fluid
BALT	Bronchus-associated lymphoid tissue
c-AMP	Cyclic AMP
CD	Clusters of differentiation
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CTRL	Control
e-NANC	excitatory-Non-adrenergic Non-cholinergic
EAR	Early asthmatic response
ECP	Eosinophil cationic protein
EDN	Eosinophil derived neurotoxin
EGF	Epidermal growth factor
E_L	Pulmonary elastance
ELAM-1	Endothelial-leukocyte adhesion molecule-1
ELISA	Enzyme-linked immunosorbent assay
ENL	Erythema nodosum lepromatous
FASR	Fast adapting stretch receptors
FBS	Fetal bovine serum
GM-CSF	Granulocyte macrophage colony stimulating factor
GP	Guinea-pig
HAT	Hypoxanthine aminopterine thymidine
HETE	hydroxyeicosatetraenoic
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
HRPO	Horseradish peroxidase
i-NANC	inhibitory-Non-adrenergic Non-cholinergic
ICAM-1	Intercellular adhesion molecule-1
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IL	Interleukin
LAR	Late asthmatic response

LFA	Lymphocyte function-associated antigens
LPS	Lipopolysaccharide
LT	Leukotriene
M	Molar
M-CSF	Macrophage colony stimulating factor
mAb	Monoclonal antibody
MALT	Mucosal associated lymphoid tissue
MBP	Major basic protein
MCP	Membrane cofactor protein
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MPP	Macrophage proinflammatory peptide
MTT	3-(4,5-dimethylthiazol-2-yl)-di-phenyltetrazolinium bromide
NA	Noradrenaline
NEP	Neutral endopeptidase
NK	Natural killer
NKA	Neurokinin A
NKB	Neurokinin B
nM	Nanomolar
NO	Nitric oxide
NOS	Nitric oxide synthase
OA	Ovalbumin
OPI	Oxalic acid-pyruvate-insulin
pAb	Polyclonal antibody
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
PG	Prostaglandin
PLA_2	Phospholipase A_2
PMA	Phytohemagglutinin
PMN	Polymorphonuclear leukocytes
RANTES	Regulated on activation, normal T-cell expressed and secreted
R_L	Pulmonary resistance
SASR	Slowly adapting stretch receptors
SCF	Stem cell factor
SP	Substance P
STD	Standard

TGF	Transforming growth factor
TK	Thymidine kinase
TNF	Tumor necrosis factor
TTX	Tetrodotoxin
VIP	Vasoactive intestinal peptide

CHAPTER 1

INTRODUCTION

PART A — AIRWAYS

Morphology

The airways are divided into two functional parts. The conducting airways of the lung refer to those airways that connect the outside world to the respiratory bronchioles and alveolar ducts. These airways deliver and remove air but are not involved in gas exchange. The lower or alveolar portion of the airways are responsible for O_2/CO_2 exchange. The conducting airways consist of the nose, pharynx, larynx, trachea, bronchi and terminal bronchioles; the respiratory portion includes the respiratory bronchioles, alveolar ducts, alveolar sacs and alveoli (Leff & Schumacker, 1993).

The right lung has three lobes (upper, middle and lower) and the left lung has two lobes (upper and lower). There are ten bronchopulmonary segments of the right lung and nine of the left lung. The left lung is smaller than the right because of the volume occupied by the heart. It has been estimated that there are 23 to 27 generations of the airways beginning in the proximal airways with the trachea and ending distally with the alveolar ducts (Leff & Schumacker, 1993).

The lungs receive blood from both the pulmonary and systemic circulations. Blood from the pulmonary circulation perfuses the capillaries in the alveolar walls for gas exchange. From the hilum to the periphery, there is a dual blood supply to the airways by both the systemic and pulmonary circulations while the large conducting airways are supplied exclusively by the bronchial systemic circulation.

Histologically, the conducting airways have the following major components to

their walls: epithelium, mucosa, submucosa, cartilage, muscle and serosal layers (from the inner lumen to the outer surface) (Bacon *et al.*, 1983). The mucosal layer is composed of a mucus blanket, airway epithelium and the basement membrane below. In the conducting airways of the lung, the epithelial layer contains ciliated pseudostratified columnar epithelial cells. The ciliary cells propel mucus from the distal to the proximal airways. The epithelium which serves as a major barrier function in the upper airways, thins to become a more permeable barrier in close continuity with a rich vascular bed in the alveoli. Gradually, the cartilage, the mucus glands and finally the airway smooth muscle are lost so that the alveoli are an efficient gas exchange system. The fluid lining the airway is composed of two layers: the periciliary fluid layer (likely resulting from active ion and water transport across the epithelia) and the mucous layer (resting atop of the fluid layer and derived from goblet cells and submucosal glands) (Djukanovic *et al.*, 1990).

Innervation

There are two groups of nerve fibers in the airways. Afferent pathways carry nerve impulses away from the sensory nerve endings and into the central nervous system (CNS). Efferent pathways carry nerve impulses from the CNS into the airways. Airway innervation is outlined in Figure 1.1.

Afferent Pathways

Sensory afferent pathways originate in the airways and travel in the vagi into the CNS. There are three types of afferent receptors in the airways:

Slowly adapting (stretch) receptors (SASR) are mechanoreceptors that act in response to tension changes across the airway wall. Cholinergic efferent innervation provides the predominant source of resting smooth muscle tone. Upon inspiration, the

reflex caused by stimulation of SASR results in inhibition of cholinergic tone. This is a classical central reflex initiated by airway afferent fibers and is called the Hering Breuer inspiration reflex; information arises from the afferent fibers, is processed in the CNS and a response is then transmitted along cholinergic efferent fibers (i.e. an inhibition of efferent activity to the diaphragm, a decrease in inspiration, and a prolongation of expiration) (Undem, 1995). These receptors are served by myelinated nerve fibers, are localized on airway smooth muscle of conducting airways and are so called because they are fast conducting and accommodate slowly to stimulus (Leff & Schumacker, 1993).

Fast acting stretch receptors (FASR) (also called irritant receptors) reside in the epithelium of large airways; they fire rapidly when stimulated, then quickly accommodate to slow firing of neural impulses. Irritants and inflammation (i.e. inflammatory mediators such as histamine) stimulate these receptors resulting in bronchoconstriction and rapid shallow breathing (Widdicombe & Wells, 1994).

Unmyelinated nerves running within the airway epithelium serve the c-fiber endings. Stimulation of these by mediators or cold air results in bronchoconstriction and mucus secretion (Widdicombe & Wells, 1994).

Thus, the afferent neurons can initiate three types of reflexes: 1) central reflexes, 2) axonal reflexes, and 3) peripheral reflexes. The Hering Breuer inspiration reflex is an example of a central reflex initiated by SASR. Central reflexes initiated by FASR and c-fibers include various defensive reflexes such as cough and mucous secretion. An axon reflex involves an action potential traveling along an afferent c-fiber (towards the CNS), then reverting back antidromically toward effector cells along collateral branches of the same axon. Neuropeptides localized to afferent c-fibers include the tachykinins and calcitonin gene-related peptide (receptor distribution and effects of these neuropeptides are described later in "Non-adrenergic Non-cholinergic Innervation").

Peripheral reflex action involves the stimulation of afferent c-fibers, and the release of transmitter (i.e. tachykinins) via an axon-reflex mechanism, in the region of local autonomic ganglion neurons. This may result in either stimulation of postganglionic fibers or a reduction in the filtering capacity of these fibers to preganglionic input from the CNS. This mechanism is relatively unstudied in human airways and likely exists in guinea-pig airways (reviewed in Undem, 1995).

Efferent Pathways

The nerve supply from the CNS to the lungs consists of classical cholinergic and adrenergic innervation as well as non-cholinergic non-adrenergic (NANC) systems (Richardson, 1979; Barnes, 1986; Barnes, 1987).

(i) Cholinergic Innervation

Cholinergic innervation is the dominant bronchoconstrictor neural pathway in the airways of humans and other animals (Richardson, 1979; Barnes, 1991a; Andersson & Grundstrom, 1987). Parasympathetic nerves consist of long preganglionic fibers coursing from the nucleus of the XI cranial nerve, through the vagus to ganglia located in the subserosal portion of the airways. The postganglionic fibers are very short. A mild and continuous degree of smooth muscle contraction is a result of tonic parasympathetic activity (Goodman & Gilman, 1990). Parasympathetic innervation is most significant in the upper airways of the lung and diminishes toward the periphery (Leff & Schumacker, 1993; Barnes, 1986). These nerves also innervate mucus glands of the airways and can increase the production of mucus glycoproteins. Acetylcholine (Ach) is the neurotransmitter released both at the ganglion and effector organ synapse, and works on nicotinic and muscarinic receptors. Nicotinic receptors are located in the ganglia and are responsible for generating action potentials on the membrane of the postganglionic nerve.

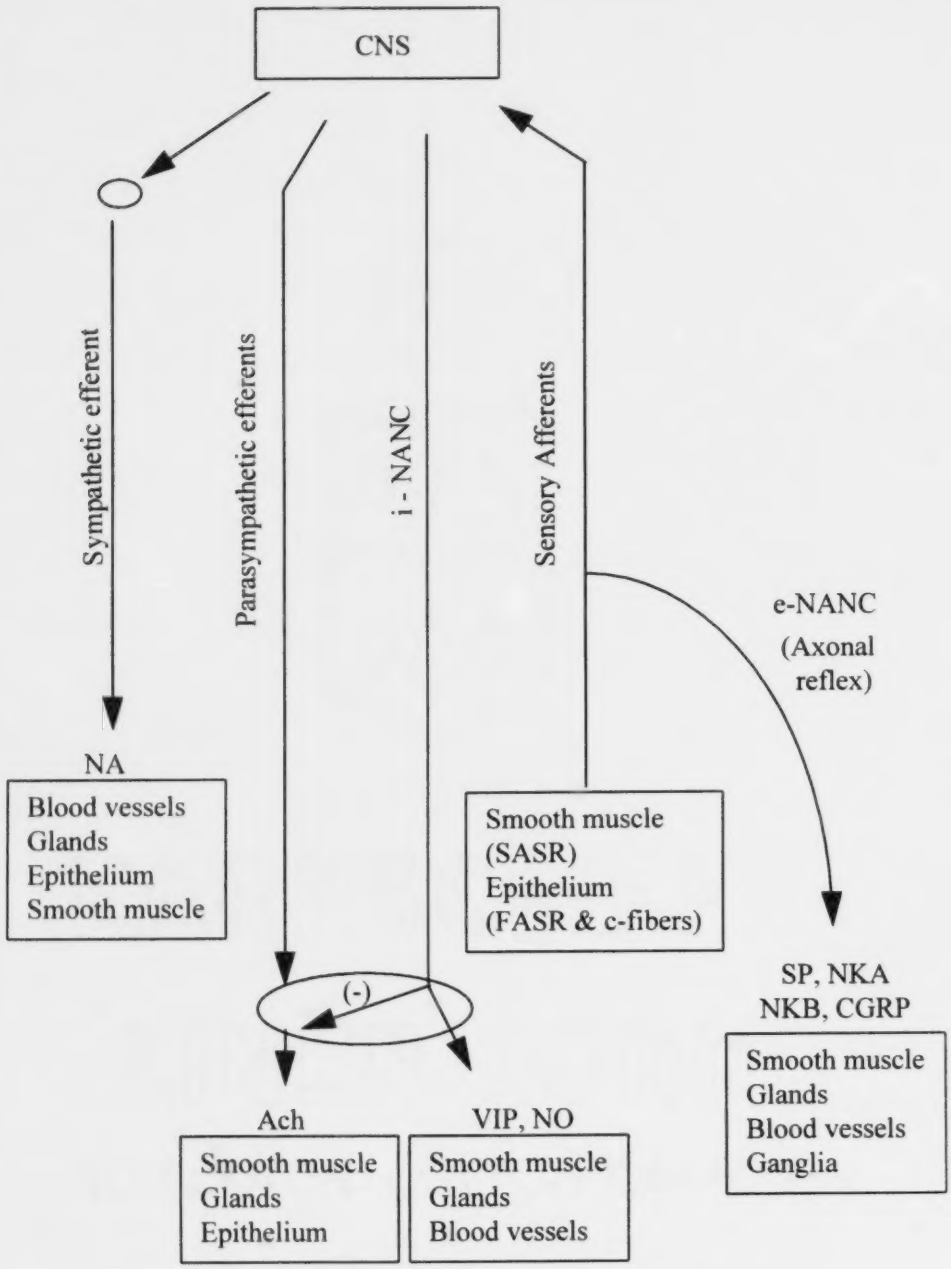

Figure 1.1. Schematic illustration of airway innervation. See thesis text for abbreviations and description. Ovals represent ganglia.

They are stimulated by nicotine or dimethylphenylpiperazinium and are blocked by hexamethonium (Goodman & Gilman, 1990). Pharmacologically, four subtypes of muscarinic receptors have been identified, while five distinct subtypes have been cloned or expressed (Barnes, 1994a). M_1 excitatory receptors are present in airway ganglia of animals; M_2 receptors are present on cholinergic nerve terminals and provide negative feedback regulation of Ach release; and M_3 receptors are found on airway smooth muscle, submucosal glands and airway epithelium, and mediate airway smooth muscle constriction, submucosal gland secretion and ion transport across the airway epithelium (Barnes, 1991a; Barnes, 1989; Barnes, 1986; Douglas, 1990; Barnes, 1994a).

(ii) *Adrenergic Innervation*

Catecholamine-containing nerve fibers exist, sparsely in the airway smooth muscle of humans (Partanen *et al.*, 1982; Barnes, 1991a). They supply submucosal glands, bronchial vessels and airway ganglia (Richardson, 1979; Boushey *et al.*, 1980; Uddman & Sundler, 1987; Barnes, 1986). The preganglionic fibers originate from the upper six thoracic segments of the spinal cord and terminate in the extrapulmonary stellate ganglia, the middle and inferior cervical ganglia and the upper four thoracic ganglia. Postganglionic fibers run in the vagi to enter the lungs on the airway walls (Barnes, 1986; Richardson, 1979). Noradrenaline (NA) is the local neurotransmitter, however circulating levels of adrenaline can activate adrenoreceptors. The two receptor types in the adrenergic system are the α- and β-adrenergic receptors. α-Adrenoreceptors are subdivided into α_1- and α_2-adrenoreceptors. The density of α-adrenoreceptors in the human lung is low; α_1-adrenoreceptors are located on submucosal glands and in the microvasculature (Barnes, 1986; Barnes, 1984). Stimulation of these receptors causes watery secretions and possibly contraction of airway smooth muscle. α_2-adreno-receptors are located presynaptically on the pre- and post-ganglionic nerves of

sympathetic and parasympathetic pathways. Their activation provides negative-feedback inhibition of noradrenaline release (Goodman & Gilman, 1990; Barnes, 1986). Post-synaptic α_2-adrenoreceptors have also been described (Barnes, 1986).

β-adrenoreceptors are divided into three subtypes; β_1-, β_2- and β_3-adrenoreceptors. Both β_1- and β_2-adrenoreceptors are found in human lung, however β2-adrenoreceptors predominate (Douglas, 1990; Barnes, 1986). β-adrenoreceptors are located on epithelial cells, endothelial cells, alveolar lining cells, macrophages, mast cells, airway smooth muscle, parasympathetic ganglia and postsynaptic terminals (Barnes, 1984; Barnes, 1986; Barnes, 1995; Insel & Wasserman, 1990; Douglas, 1990). β-agonists such as salbutamol cause smooth muscle relaxation through the β_2-adrenoreceptor. In the trachea and bronchi, β-adrenoreceptors have an inhibitory effect on the firing of parasympathetic ganglion cells (likely a B_2-adrenoreceptor) (Nijkamp, 1993; Barnes, 1995). Thus, β-Adrenoreceptor activation has been shown to stimulate mucus secretion, inhibit mast cell mediator release and modulate parasympathetic transmission (Barnes, 1986; Insel & Wasserman, 1990; Barnes, 1991a; Kneussl & Richardson, 1978; Nijkamp, 1993). Relaxant responses to catecholamines that are resistant to blockade by α- and β-adrenoreceptor antagonists have been observed in gastrointestinal smooth muscle preparations, skeletal muscle and heart of some species. These atypical β-adrenoreceptors represent the β_3 subclass of receptors (Szentivanyi, 1994).

(iii) *Non-adrenergic Non-cholinergic Innervation*

Neurally-mediated responses, that are not blocked by adrenoreceptor or cholinoreceptor antagonists have been described in the airways of several animal species including humans and guinea-pigs (Barnes *et al.*, 1991a). These are thus termed non-adrenergic, non-cholinergic and consist of both excitatory (e-NANC) and inhibitory

(i-NANC) responses. The i- and e-NANC nerve fibers travel in the vagi, enter the lungs and relay in the parasympathetic ganglia together with cholinergic nerves. The neuropeptides released from these nerves occur also as co-transmitters with adrenergic, cholinergic and sensory nerves (Barnes, 1986, Barnes, 1990). The interactions are complex; some mechanisms are described briefly in "Afferent Pathways" section, p.2.

In animal airways, e-NANC fibers lie close to bronchial epithelium, smooth muscle, mucus glands and blood vessels (Lundberg *et al.*, 1984; Polak & Bloom, 1983), and activation of e-NANC nerves within the lungs induces a number of responses which include mucus secretion, vasodilatation, plasma exudation and smooth muscle contraction (Barnes, 1987b). These nerves consist of the c-afferent sensory fibers, and are sensitive to capsaicin (the pungent principal of hot *Capsicum* peppers) and tetrodotoxin (TTX) (Holzer, 1991; Andersson & Grundstrom, 1983).

The excitatory mediators released from these nerves include the tachykinins, namely substance P (SP), neurokinin A (NKA), neurokinin B (NKB) (in some species), and calcitonin gene-related peptide (CGRP) (Barnes *et al.*, 1991). These sensory neuropeptides may be released locally, as a result of local axonal reflexes or in conjunction with cholinergic stimulation (Barnes, 1986). They produce their biological effects through activation of specific receptors (Lundberg *et al.*, 1983; Barnes, 1987; Norris *et al.*, 1990). Three receptor types exist for SP, NKA and NKB, according to their relative potency to bind tachykinins. NK-1, NK-2 and NK-3 receptors bind with highest affinity to SP, NKA and NKB respectively (Frossard & Advenier, 1991). Potent and selective antagonists for each of the receptor subtypes have been developed, allowing for future investigation of tachykinin effects in the airways. In isolated human bronchi, tachykinins produce concentration-dependent contractions with the order of potency NKA>>SP>>NKB (Frossard & Barnes, 1991). NK-2 receptors are thus likely

involved in bronchial smooth muscle contraction in this system. The NK-1 receptor and thus SP have been shown to be the primary mediator of mucus secretion in the human bronchi (Rogers *et al.*, 1989). SP has also been shown to activate human skin mast cells (Frossard & Advenier, 1991; Foreman *et al.*, 1983), murine peritoneal mast cells (Frossard & Advenier, 1991; Barnes *et al.*, 1986) and cause the proliferation of T cells (Payan *et al.*, 1983; Payan & Goetzl, 1987). CGRP constricts human smooth muscle, *in vitro*, and may act synergistically with SP to cause vasodilation (Barnes, 1987). Treatment of animals with capsaicin releases SP and NKA from sensory nerves acutely and chronic or high dose administration of capsaicin depletes the lung of these neuropeptides (Lundberg *et al.*, 1983; Theodorsson-Nerheim *et al.*, 1985). These properties of capsaicin have been exploited as a means to investigate the role of these nerves in the airways. Capsaicin acts through activation of a non-selective cation channel. Sodium then enters the neuronal cell against its concentration gradient, causing depolarization of the cell, action potential generation and the release of mediators (O'Neill, 1991).

In the human lung, the i-NANC system is the only neural bronchodilating mechanism. In animals, stimulation of the vagus in the presence of adrenergic and cholinergic blockade results in long-lasting bronchodilation which may be inhibited by ganglion blockers (Diamond *et al.*, 1980; Irvin *et al.*, 1980). Vasoactive intestinal peptide (VIP) is thought to be the primary mediator of the i-NANC system (Barnes, 1987; Barnes, 1990). However there is increasing evidence that nitric oxide (NO) may play a role, especially in humans. NO may also serve as a braking mechanism for cholinergic transmission (Tucker *et al.*, 1990; Li & Rand, 1991; Belvisi *et al.*, 1991; Lei *et al.*, 1993; Kuo *et al.*, 1992; Nijkamp & Folkerts, 1994). An inhibitor of nitric oxide synthase (NOS) blocks the TTX-sensitive (i.e. neural) dilator response to neural stimulation in human tracheal rings from normal donors (Belvisi *et al.*, 1992).

Histochemical studies show VIP-immunoreactive nerves are present in the larger airways, diminish in the smaller airways and are virtually absent from bronchioles. They are located in airway smooth muscle, submucosal glands, airway parasympathetic ganglia and blood vessel walls (Uddman & Sundler, 1987; Barnes, 1990; Dey *et al.*, 1981). Autoradiographic studies show that VIP-receptors are present on airway glands, epithelium and vascular smooth muscle (Carstairs & Barnes, 1986; Uddman & Sundler, 1987). VIP is a potent vasodilator (Lundberg *et al.*, 1984), a potent stimulator of mucus secretion (Peatfield *et al.*, 1983; Peatfield & Richardson, 1983) and relaxes human bronchi *in vitro* (Palmer *et al.*, 1986).

Normal Immune Responses in the Lungs

Normal defense systems within the lung include mechanical (filtration, cough and mucociliary clearance), phagocytosis and immune mechanisms (both humoral and cell-mediated). Most antigenic material deposited in the airways is efficiently removed by mechanical and phagocytic clearance mechanisms.

Lymphatic vessels drain both the airway mucosa and the pulmonary paryenchyma and are thus classified as pleural lymphatics (which are present in the loose connective tissue that underlies the pleural lining cells) and the peribronchovascular lymphatics (which surround both the bronchial and vascular trees) (Morrow, 1971; Kaltreider, 1991). Lymphocytes that enter lung interstitium from pulmonary capillaries may traverse lymphatic channels to the hilar lymph nodes (the regional draining lymph nodes of the respiratory tract). Specialized lymphoid tissue distributed throughout the tracheobronchial tree is called "bronchus-associated lymphoid tissue" (BALT), and is essential in pulmonary defense. It consists of follicular lymphoid tissue BALT is more abundant in some animal species such as rodents and rabbits while human airways contain lesser amounts (Bienenstock, 1984). Other mucosal surfaces possess this

specialized lymphoid tissue and it is therefore more generally termed MALT or "mucosal-associated-lymphoid tissue".

(i) *Bronchus-Associated-Lymphoid Tissue*

Lymphoid tissue at mucosal surfaces such as the airways can be divided into four levels. These are (1) lymph nodes; (2) lymphoid nodules; (3) lymphoid aggregates and (4) individual or small clusters of cells within the interstitial and alveolar spaces (Kaltreider, 1991). In order to initiate a primary response, antigen within the airways must cross the epithelial surface to come in contact with the lymphoid tissue in the lamina propria. This is facilitated by specialized cells called M-cells (so designated because of the microfolds that cover their surface) that lie within the epithelium and overlie the lymphoid nodules or follicles. M-cells bind antigen on the luminal side, endocytose it, transport the endocytic vesicles to the opposite side, and exocytose them, releasing the antigen to the underlying lymphoid tissue. M-cells cannot present antigen. Antigen is then taken up by antigen presenting cells (APC) (such as dendritic cells or alveolar macrophages), to be processed and presented to reactive B- and T-lymphocytes, thus initiating both cellular and humoral responses. APC may present antigen to lymphocytes juxtaposed to the M-cells or they may migrate to the lymph nodes to do so. (reviewed in Goldie, 1994; Bienenstock, 1984).

(ii) *Cellular and Humoral Immune Response*

Receptors on $CD4^+$ T-lymphocytes recognize antigen and Class II MHC molecules on the surface of APC. This interaction between the two cells causes the activation of the T-lymphocyte, the liberation of interleukin-1 (IL-1) and IL-2, and the expression of IL-2 surface-membrane receptors. Activated $CD4^+$ T-lymphocytes undergo clonal expansion and differentiation into effectors of delayed-type

hypersensitivity, and into inducers of the proliferation and differentiation of B-lymphocytes, cytotoxic lymphocytes (CTL) and T-suppressor cells.

B lymphocytes are themselves antigen-presenting cells. Thus antigen specific B cells can bind protein antigens via membrane-bound immunoglobulin and internalize, process and present MHC II associated peptide determinants to specific $CD4^+$ helper T-lymphocytes. The physical contact between activated helper T cells and B cells initiates B cell responses (i.e. clonal expansion and secretion of antigen specific antibody). Helper T cells (i.e. $CD4^+$ cells) secrete cytokines that stimulate the further proliferation and differentiation of the B cell. Appropriate presentation of antigen therefore induces local clonal expansion of both T and B effector cells. Thus, defense mechanisms within the lung are a collaborative consort of both humoral and cell mediated events (reviewed in Goldie, 1994, and Abbas *et al.*, 1994).

Within the bronchial secretions, all major classes of immunoglobulin (Ig) have been detected, dominated collectively by the concentration of IgA dimer (Young & Reynolds, 1984). The concentration of IgG relative to that of IgA increases as one proceeds distally to the lower respiratory tract (Young & Reynolds, 1984). In bronchoalveolar lavage (BAL) fluid, concentrations of IgG considerably exceed that of IgA. Except for IgA which is the major isotype of antibody that can be actively transported through epithelia, the origins of immunoglobulins in the lung are generally from exudation out of serum (Out *et al.*, 1991). Dimeric IgA is produced by plasma cells in the lamina propria and binds to secretory component on the basal and lateral surfaces of epithelial cells. This covalently bound complex is endocytosed into the epithelial cells and transported in vesicles to the luminal surface. The extracellular domain of the secretory component is then proteolytically cleaved, releasing IgA into the bronchial lumen. IgA then serves to neutralize microbes and toxins, agglutinate microbial

organisms for efficient excretion, block the uptake of antigens, and inhibit the adherence of microbes to mucosal surfaces. IgG and IgM efficiently agglutinate particulate antigens, activate complement and neutralize certain bacteria and viruses. IgG antibody is most effective at opsonization and IgE antibody also assists in antibody-dependent cell-mediated cytotoxicity (ADCC) against certain parasitic and viral infections (reviewed in Abbas *et al.*, 1994).

PART B — ASTHMA

Introduction and Definition of Asthma

The National Institutes of Health executive summary on the guidelines for the diagnosis and management of asthma (1991) describes asthma as a lung disease with the following characteristics: airway obstruction (that is reversible), airway inflammation and airway hyperresponsiveness to various stimuli. Asthma can be classified into two groups: extrinsic (allergic or atopic) and intrinsic (idiosyncratic or non-atopic). Extrinsic asthma is characterized by increased serum levels of IgE and a positive wheel-and-flare reaction to intradermal injections of antigen extracts normally found in the air . Patients with intrinsic asthma do not have a personal history of allergy, have normal IgE levels and have negative skin reactions to allergens (Weiss & Stein, 1993).

The development of nonspecific airway hyperresponsiveness as shown by increases in both the sensitivity and magnitude of airway constriction to various stimuli (cold air, exercise, pharmacological compounds, irritants, appropriate antigen) is characteristic of asthma. After exposure to the appropriate stimulus, this hyper-responsiveness generally manifests itself in two clinical phases. The early asthmatic response (EAR) starts within 10 - 15 min post exposure to stimuli, and recovers within 1 - 3 h. The late asthmatic response (LAR) begins 3 - 4 h post exposure to stimuli and

peaks 8 - 12 h later. It is characterized by prolonged, but less severe broncho-constriction. Fifty to sixty percent of extrinsic (allergic) asthmatics are dual responders and the remainder elicit either an EAR or a LAR (reviewed in Weiss & Stein, 1993).

The Pathology of Asthma

The pathology of asthma is described in many reviews (Arm & Lee, 1991; Jeffrey, 1992; Hogg, 1993; Beasley *et al.*, 1993). Briefly, asthmatic airways have the following features:

Airway eosinophilia beneath the mucosal epithelium and in the alveolar spaces may be a characteristic feature of asthma (Laitinen & Laitinen, 1988; Beasley *et al.*, 1989). In patients with mild asthma, blood eosinophilia may be normal, however ongoing disease is associated with eosinophils or eosinophil-derived basic proteins in the blood sputum or BAL fluid (Bruynzeel *et al.*, 1987; Bousquet *et al.*, 1990; Kroegel *et al.*, 1991; Kroegel *et al.*, 1992).

Epithelial damage and shedding is a characteristic feature of fatal *status asthmaticus* and epithelial derangement ranging from detachment of the epithelial cell layer from the basal cell layer, to sloughing of the entire epithelium is usually present in asthmatic airways (Laitenen, 1985; Davies & Devalia, 1991). Typically, ciliated epithelial cells are swollen and the intercellular spaces widened; mucous cells and basal cells proliferate while ciliary cells are lost from the epithelium in response to injury (Laitinen & Laitinen, 1991).

Epithelial basement membrane thickening has been classically described in the airways of asthmatics (referring to a prominent hyaline-like layer situated under the basal lamina). Recent immunohistochemical and electron microscopic studies however (Roche *et al.*, 1989; Gabbrielli *et al.*, 1994), show that the bronchial epithelial basement

membrane is of normal thickness in asthma, but there exists a dense deposition of collagen fibrils in the subepithelial region and is a result of fibroblast activation rather than bronchial epithelial cell dysfunction (Alexander *et al.*, 1991).

Mucosal plugging of the bronchioles is a common feature of deaths from asthma (Djukanovic *et al.*, 1990). This is due to hypertrophy and hyperplasia of submucosal glands and an increased number of goblet cells in the cartilaginous airways of asthmatic patients (Dunnill, 1960). Secretions from the airways of asthmatics, contain eosinophils, Charcot-Leydon crystals and shed epithelial and inflammatory cells (sometimes in the form of clumps referred to as Creola bodies) (Djukanovic *et al.*, 1990). Changes in the tracheobronchial microvasculature (i.e. the production of microvascular leakage by such mediators as histamine) causes edema to the tracheobronchial mucosa which contributes towards airway narrowing (Persson, 1986).

Hypertrophy and hyperplasia of airway smooth muscle has been described in the airways from asthmatics and in animal models of asthma (Dunnill *et al.*, 1969; Ebina *et al.*, 1993; Sapienza *et al.*, 1991).

The Pathogenesis of Asthma

The exact pathogenesis of asthma is unknown. Models of mechanical, neuronal or cellular abnormalities have been proposed to explain the pathological features of asthma (Barnes *et al.*, 1988; Barnes, 1991a). It is likely that all these mechanisms contribute to the perpetuation of the pathology observed in asthma.

Mechanical

The epithelium functions as a diffusion barrier and thus epithelial damage may contribute to airway hyperresponsiveness by allowing external stimuli access to airway

smooth muscle and sensory nerve endings (Sparrow & Mitchell, 1991). Activation of sensory nerve endings or irritant receptors on smooth muscle could result in central reflex bronchoconstriction. Also, epithelial cells modulate neuropeptides secreted from c-afferent fibers, by being a source of neutral endopeptidase (NEP), an enzyme that degrades and inactivates SP, NKA and VIP (Flavahan *et al.*, 1985). Loss of NEP can occur with epithelial damage. Tracheal strips that have been stripped of epithelium but with smooth muscle intact (ferret trachea) are much more sensitive to bronchoconstrictor actions of SP (Sekizawa *et al.*, 1987), presumably in part due to the lack of NEP.

Other studies have described decreases in airway baseline caliber as being fundamental to the pathogenesis of asthma and airway hyperreactivity. The internal diameter of the airways is reduced by increased secretion of mucus into the airways, increases in airway thickness due to mucosal edema, inflammation and fibrosis, and airway smooth muscle hypertrophy and hyperplasia (Gabbrielli *et al.*, 1994; Jeffery, 1992; Barnes *et al.*, 1988; Lambert *et al.*, 1993).

Alterations in airway smooth muscle could also account for nonspecific airway hyperresponsiveness. *In vitro* studies show hyperresponsiveness of airway smooth muscle from asthmatic patients (de Jongste *et al.*, 1987; Schellenberg & Foster, 1984). This could be secondary to inflammation.

Neuronal

Cholinergic nerves are the dominant neural bronchoconstrictor pathway in animal and human airways (Richardson, 1979; Barnes, 1986). An increase in vagal drive has been suggested in asthma by the observation of enhanced vagal cardiac tone in asthmatic patients (Kallenbach *et al.*, 1985). As described above, it is also speculated that in the presence of airway epithelial damage, mediators released by activated epithelial and mast

cells or mechanical irritation could stimulate sensory c-afferent fibers and irritant receptors, leading to reflex bronchoconstriction (Kaufman *et al.*, 1980; Coleridge & Coleridge, 1984). Theories of increased acetylcholine release via defects in β-adrenoreceptor or α_2-adrenoreceptor negative feedback mechanisms, or increases in cholinergic airway smooth muscle stimulation due to increases in muscarinic receptor density, affinity or efficiency of signal transduction have been investigated but the evidence is not conclusive (reviewed in Barnes, 1991a).

Adrenergic mechanisms may also be involved. β-agonists reverse bronchospasm in asthmatic airways. β-adrenergic blockade increases airway responsiveness in asthmatic subjects (Townley *et al.*, 1976), however it does not produce airway hyperresponsiveness or asthma in normal subjects (Richardson & Sterling, 1969; Tattersfield *et al.*, 1973; Zaid & Beall, 1966). Alpha-adrenergic responsiveness may be increased in asthma (Szentivany, 1968). In response to histamine and serotonin, α-adrenoreceptor-mediated constriction of canine trachea has been demonstrated (*in vitro* and *in vivo*) (Kneussl & Richardson, 1978: Barnes *et al.*, 1983; Brown *et al.*, 1983). Again however, α-blockers such as prazosin have little or no effect in asthma (Barnes *et al.*, 1981; Baudouin *et al.*, 1988).

Defects in the e- and/or i-NANC neuronal system or the peptides they release, may account for airway hyperresponsiveness. Mast and other inflammatory cells release proteases and peptidases (i.e. tryptase) that degrade proteins like VIP. VIP is a bronchodilator and a co-transmitter with acetylcholine in airway cholinergic nerves (Laitinen *et al.*, 1985) and may act as a down-modulator of cholinergic neurotransmission. The activation of e-NANC systems releases pro-constrictor substances and is discussed above.

Cellular

Viewing asthma as an inflammatory disease has taken favour in recent years (reviewed in Kay, 1991; Bousquet *et al.*, 1991; Barnes, 1987; Leff *et al.*, 1991). With the advent of bronchoscopy and bronchial biopsy it has become evident that inflammation is an existing component of asthma even in mild disease (Beasley *et al.*, 1989; Jeffrey *et al.*, 1989). Furthermore, airway inflammation in asthma is described as a complex multicellular event, leading to morphological changes that may be the driving force in the development of bronchial hyperresponsiveness (Busse & Reed, 1993; Reid *et al.*, 1989). Only recently has the influence of inflammatory mediators on airway innervation been under investigation. There is growing evidence that inflammatory mediators may activate afferent nerve fibers. Studies on guinea-pig bronchial ganglion neurons indicate that exogenously applied inflammatory mediators typical of those released during antigen challenge, can increase the excitability of ganglion neurons. This increases the amplitude of excitatory postsynaptic potentials and, consequently, decreases the filtering capacity of the ganglia (Myers *et al*, 1991; Undem *et al*, 1993). Other mechanisms may involve increases in the excitability of nerve endings rendering them more responsive to other stimuli, or alterations in the amount of transmitter released at neuro-effector junctions (reviewed in Undem, 1995).

Inflammatory cells found in the lungs and the mediators they secrete are discussed in this section.

(i) *Cells*

Mast cells are located in the lumen of the airways, in the bronchial epithelium in the submucosa and in the lung parenchyma (Kay, 1991). Mast cells have been found in increased numbers in BAL of both atopic and nonatopic asthmatic subjects (Kirby *et al.*,

1987; Mattoli *et al.*, 1991). Activation of mast cells and thus the liberation of mediators may occur via exercise, cold air, ventilation or allergen exposure. Upon stimulation or (FcεR) cross-linkage, human lung mast cells release histamine, chymase, tryptase and tumor necrosis factor alpha (TNFα) from intracellular storage granules. Lung mast cells also elaborate LTC_4, PGD_2, PAF, various chemotactic peptides and proteoglycans (Kay, 1991; Alexander *et al.*, 1991; Djukanovic *et al.*, 1990). Mast cells also produce several cytokines including IL-3, IL-5, IL-6 and TNFα (Gordon *et al.*, 1990).

There is much evidence implicating the eosinophil as a major effector cell in asthma. Eosinophils are present in increased numbers in the lavage fluid of allergic and non-allergic asthmatic subjects (reviewed in Smith & Deshazo, 1993). They are recruited into the lung by chemotactic factors released from mast and other cells including T-lymphocytes. These factors include PAF, LTB_4 and cytokines (TNFα, IL-1, IL-3, IL-5 and granulocyte macrophage colony stimulating factor (GM-CSF)) (Busse & Sedgwick, 1992; Reed, 1994; Makino & Fukuda, 1995). The eosinophil is a source of many mediators that can produce epithelial damage, mucus production, edema and bronchospasm. Many of these are liberated from intracellular storage granules and include major basic protein (MBP) and eosinophil cationic protein (ECP). ECP and MBP are both cytotoxic to the respiratory epithelium *in vitro* and in experimental animals (Gleich *et al.*, 1979) and may contribute to the denudation of the epithelium observed in asthma (Kay, 1991). Other mediators secreted by eosinophils are LTC_4, LTD_4, LTE_4 and PAF (Chung, 1986; Lee, 1987; Gleich & Adolphson, 1991). As well, blood and lung eosinophilia is seen in the LAR and correlates with disease severity in asthmatic patients (De Monchy *et al.*, 1985; Lam *et al.*, 1987; Wardlaw *et al.*, 1988). Eosinophils are a source for TNFα, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6 and transforming growth factor alpha and beta (TGFα and β) (Barnes, 1994).

Recent studies have indicated that airway epithelial cells can synthesize and release cytokines (i.e. IL-6, IL-8, GM-CSF, TNFα, platelet derived growth factor (PDGF)), lipid mediators, and reactive oxygen species in response to a number of pathologically relevant stimuli. This may result in the expression of adhesion molecules for inflammatory cells, thereby contributing to inflammation (reviewed in Adler *et al.*, 1994; Raeburn & Webber, 1994; Barnes, 1994b).

Neutrophils reside in the larger airways of both normoresponsive and hyperresponsive individuals (Kay, 1991). Their role in asthma is unclear. Studies indicate increased percentages of neutrophils in the lavage from patients with stable asthma, while others do not (reviewed in Smith & Deshazo, 1993). Neutrophils are a potential source of a wide variety of mediators which include cytokines, oxygen metabolites, proteases, cationic material, prostaglandins, thromboxanes, LTB_4 and PAF (Djukanovic *et al.*, 1990). Studies indicate that peripheral blood neutrophils become "activated" after allergen- and exercise-induced early and late phase asthmatic responses (Kay, 1991; Moqbel *et al.*, 1986; Carroll *et al.*, 1985, Durham *et al.*, 1984; Papageorgiou *et al.*, 1983).

T-lymphocytes ($CD4^+$ and $CD8^+$) are increased in the BAL fluid from asthmatic subjects (Kelly *et al.*, 1989). Also, activated T-lymphocytes are present in the bronchial biopsy of human subjects with asthma, while numbers are significantly lower in non-asthmatics (Azzawi *et al.*, 1989). The activation of T-lymphocytes and subsequent eosinophil recruitment and activation may contribute to epithelial damage and possibly also to bronchial hyperresponsiveness in asthma (Anderson and Coyle, 1994; Agostini *et al*, 1993; Jolles *et al.*, 1993). In biopsies from asthmatics, the numbers of activated T-lymphocytes could be correlated with the total number of eosinophils, as well as the number of activated eosinophils. Furthermore, the degree of activation could be

correlated with disease severity, as assessed by measurement of bronchial hyper-responsiveness (Corrigan & Kay, 1993).

In humans and mice, $CD4^+$ lymphocytes can be divided into two types, TH_1 and TH_2, based upon the pattern of cytokines that they produce (Mosmann & Coffman, 1989; Del Prete, 1992). TH_1 cells secrete IL-2, interferon gamma (IFNγ), and TNFβ, but not IL-4, IL-5 or IL-6. TH_2 cells secrete IL-3, IL-4, IL-5 and IL-6, but not IL-2, IFNγ and TNFβ. Both cell types secrete other cytokines including GM-CSF and TNFα (Del Prete, 1992, Corrigan & Kay, 1993). IFNγ, when added *in vitro* favours the expression of TH_1 type cells and IL-10 or IL-4 (secreted by TH_2 cells) inhibits TH_1 clone proliferation, probably via an effect on antigen presenting cells (Corrigan & Kay, 1993). IL-4, and IL-5 favor the growth, differentiation and activation of mast cells and eosinophils, as well as IgE production from B-cells (Coffman & Carty, 1986; Del Prete *et al.*, 1988; Saito *et al.*, 1988, Clutterbuck *et al.*, 1989; Leung & Geha, 1987). IL-3, IL-6 and GM-CSF are active in the control of eosinophil production in the bone-marrow (Alexander *et al.*, 1991). The different cytokine profiles are associated with very distinct functions of the two types of T-lymphocytes. In general, TH_2 cytokines aid in enhancing B cell secretion of immunoglobulin, particularly IgE. TH_1 cells also provide help to B cells, but function also to induce delayed-type hypersensitivity reactions. This has led to the hypothesis that in diseases of atopy, such as asthma, both production of IgE and eosinophilia are probably the result from the expansion of TH_2-like cells and/or their preferential accumulation in target organs (Anderson & Coyle, 1992; Del Prete, 1992; Holgate, 1995). Further effects of cytokines are covered in later sections.

Pulmonary macrophages are detailed in later sections.

(ii) *Inflammatory Mediators*

Histamine is located in the granules of mast cells and basophils. Histamine induces bronchoconstriction and microvascular leakage via H_1 receptors (Evans *et al.*, 1989; Finney *et al.*, 1985). The role of H_2 receptors in human airways is less certain, but they are likely immunoregulatory. In guinea-pig lung, H_2 receptors mediate bronchodilation (Chand & De Roth, 1979). Activation of H_3 receptors results in an inhibitory effect on airway cholinergic and NANC nerves (Ichinose & Barnes, 1989a, 1989b; Ichinose *et al.*, 1989).

The lipid-derived mediators refers to the metabolites of arachidonic acid (AA), a 20 carbon unsaturated fatty acid found in the cell membrane. Prostaglandins (PG) are formed when AA is oxidized by cyclooxygenase to the cyclic endoperoxidases, PGG_2 and PGH_2. A series of other enzymatic pathways leads to the formation of PGD_2, E_2, $F_{2\alpha}$, I_2 and thromboxane A_2 (TxA_2). PGD_2, $F_{2\alpha}$ and TxA_2 contract airway smooth muscle *in vitro*, whereas PGE_2 and PGI_2 weakly dilate airway smooth muscle (Gardiner, 1986; Barnes, 1987a; Barnes *et al.*, 1991a); PGD_2 and $F_{2\alpha}$ increase mucus secretion, and are chemokinetic for neutrophils and eosinophils (Barnes *et al.*, 1991a; Barnes, 1991). Leukotrienes (LT), hydroxyeicosatetraenoic acid (HETE)s and lipoxins are formed when AA is oxidized by the lipoxygenase pathway. Together, LTB_4, C_4 and D_4 are referred to as "slow reacting substance of anaphylaxis". They induce mucus secretion, airway smooth muscle contaction and microvascular leakiness (Dahlen *et al.*, 1980). LTB_4 is a potent chemokine for neutrophils and to some extent for eosinophils. In addition, 15-HETE and lipoxin A_4 have been shown to have bronchoconstrictor effects. Platelet-activating factor (PAF), is formed also from phospholipids within the

cell membrane, by the action of phospholipase A_2 (PLA_2). PAF is a potent broncho-constrictor in experimental animals and humans (Page, 1990; Barnes *et al.*, 1991b). It also induces mucus secretion, microvascular leakiness and activates a wide range of inflammatory cells, particularly eosinophils (Barnes *et al.*, 1991b). Importantly, PAF induces a non-selective and long-lasting airway hyperresponsiveness in animals (monkeys, guinea-pigs, humans) (Patterson *et al.*, 1984; Mazzoni *et al.*, 1985; Chung *et al.*, 1986; Cuss *et al.*, 1986).

Neuropeptides such as SP, NKA, NKB, and CGRP, contained in sensory nerves, are mediators of neurogenic inflammation. In humans, SP, NKA and CGRP release may cause bronchoconstriction, vasodilation, plasma exudation and mucus secretion. Sensory nerves may become sensitized by inflammatory products and triggered by mediators such as bradykinin, resulting in exaggerated inflammation. The effects of SP, NKA and CGRP may be further amplified by loss of the major degrading enzyme, NEP, from epithelial cells (Barnes, 1991).

Kinins, adenosine, complement, serotonin, and oxygen radicals and may also have a role in the inflammatory processes of asthma (Barnes *et al.*, 1991b).

Cytokines, are pro-inflammatory mediators that are released by many cell types including mast cells, eosinophils, T-and B-lymphocytes, macrophages, epithelial cells, endothelial cells and fibroblasts. They have been implicated in the pathophysiology of asthma. Cytokines are important in producing IgE, the eosinophilic inflammation of asthma, and the structural changes that are a consequence of chronic inflammation. Table 1.1 summarizes the predominant cytokines in the lung, their cellular source and some of their biological effects that are relevant to allergy (information collected from Barnes, 1994a; Bittleman and Casale, 1994; Anderson and Coyle, 1994; Howarth, 1995; Agostini *et al.*, 1993).

In the context of asthma, cytokines play an integral role in the coordination of the allergic response and the persistence of inflammation. Figure 1.2 portrays the complex interactions between cells and cytokines involved in allergic disease. The following is a brief description of these cytokine interactions:

Human airway mast cells generate and store preformed cytokines such as IL-3, IL-4, IL-5, IL-6, GM-CSF and TNFα (Bradding *et al.*, 1994; Ackerman *et al.*, 1994). Also, there is increased mast cell expression of TNFα and secretory IL-4 in asthma (Bradding *et al.*, 1994). Upon mast cell activation by inhaled antigen, these stored products may be released, affecting and activating epithelial cells, airway smooth muscle, B- and T-lymphocytes and macrophages. IL-4 and IL-5, also released by other immunocompetent cells (i.e. T-lymphocytes), play critical respective roles in the switching of B-lymphocytes to produce IgE (Romagniani, 1990), and in eosinophilic infiltration (Sanderson, 1992).

TABLE 1.1

Cytokines and the Allergic Response

Response	Cytokine	Primary Source	Activity
IgE regulation	IL-4	Mast cells, TH_2 cells	Induces IgE isotype switching
	IL-2, IL-5, IL-6, IL-13	TH_2 cells	Synergizes with IL-4
	IFNγ	Macrophages, TH_1, NK cells	Antagonizes some IL-4 effects
Eosinophilia	IL-3, IL-5, GM-CSF	Mast cells, T cells, eosinophils	Stimulates eosinophil growth
Mast cell development and activation	IL-3, IL-5, IL-9, IL-10, SCF	Mast cells, macrophages, T cells	Stimulates mast cell growth
Inflammation	IFNγ, GM-CSF, G-CSF, TNFα, IL-1, IL-4, IL-6, IL-8	Macrophages, T cells, mast cells, eosinophils epithelial cells	Activates neutrophils
	RANTES, GM-CSF, TNFα, IL-1, IL-3, IL-5	Macrophages, T cells	Activates eosinophils
	IFNγ, TNFα, M-CSF, GM-CSF, IL-1, IL-2, IL-3, IL-4	T cells, macrophages, eosinophils, mast cells	Activates macrophages
	TNFα, IL-1, IL-2, IL-6	Macrophages, mast cells, epithelial cells	Activates T-lymphocytes
	PDGF, TGFβ, IL-8 FGF, GM-CSF	Epithelial cells	Activates fibroblasts
	TNFα, IL-1, IL-6	Mast cells, macrophages	Activates epithelial cells

Summary of predominant cytokines in the lung, their cellular source and their biological effects that are relevant to allergy.

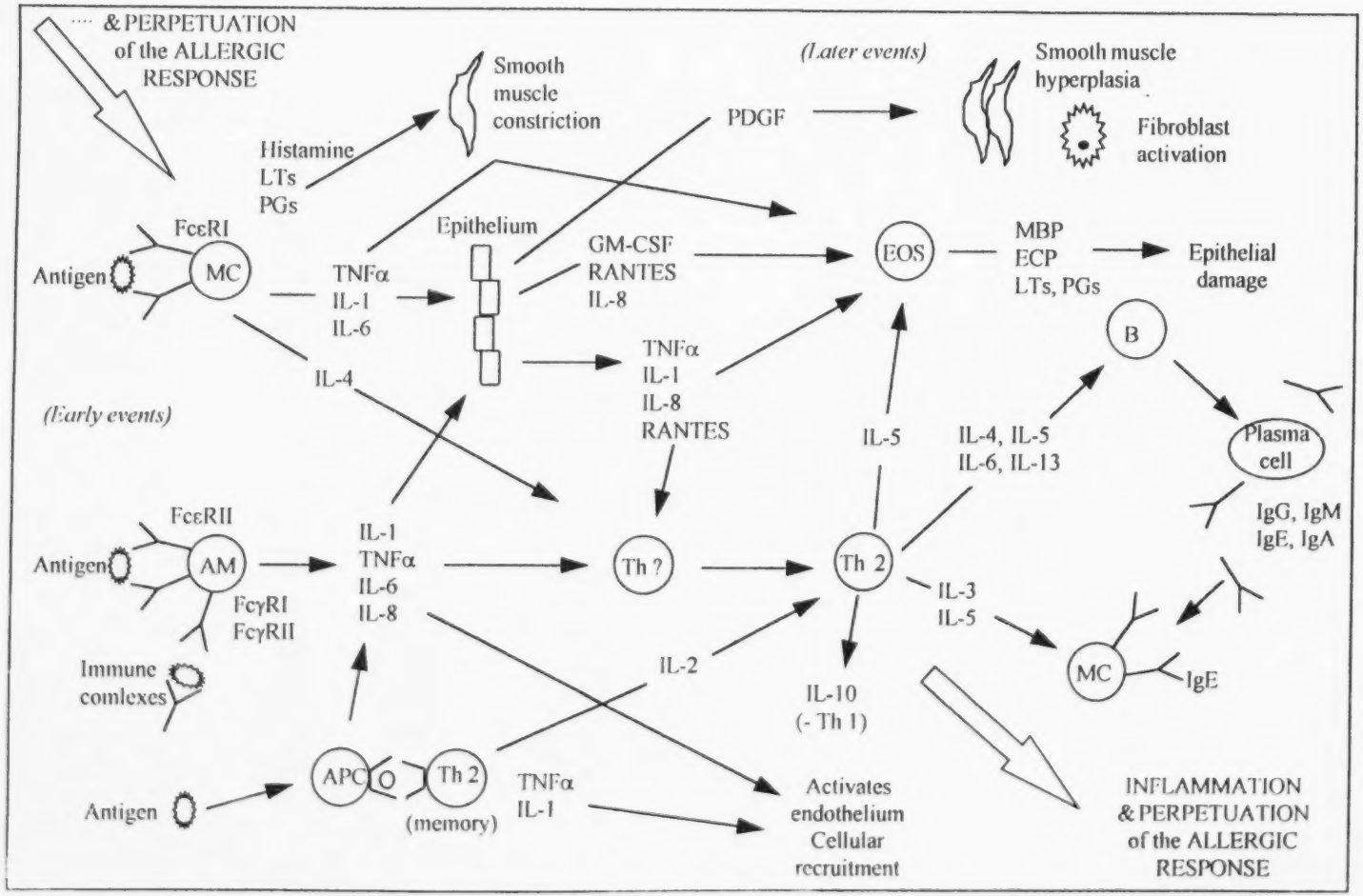

Figure 1.2. Schematic illustration showing the complex interactions between cells and cytokines involved in asthma.

Alveolar macrophages can be activated by exposure to inhaled antigen (via Fc epsilon II receptors (FcεRII)) to release IL-1, TNFα and IL-6 (Gosset *et al.*, 1991; Gosset *et al.*, 1992). These cytokines may then act on epithelial cells to release a second wave of cytokines (GM-CSF, RANTES (regulated on activation normal T-cell expressed and secreted), IL-8) which amplify the inflammatory response and are chemotactic for cells such as eosinophils. Eosinophils are also capable of releasing cytokines (IL-3, IL-5, GM-CSF), that may work by an autocrine mechanism to enhance eosinophil survival (Barnes, 1994a). The initial release of TNFα and IL-1 from macrophages (and TNFα from mast cells) increases the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) on airway epithelium and vascular endothelium, leading to the adherence of leukocytes at those surfaces (Barnes, 1994a; Tosi *et al.*, 1992). Macrophages also process exogenous protein antigens and thus act as antigen-presenting cells for T-lymphocytes. In this role, they release cytokines such as TNFα, IL-1, IL-6, IL-8, GM-CSF and IFNγ, which also contribute to airway inflammation by the recruitment and activation of neutrophils, eosinophils and more T-lymphocytes.

Following antigen recognition, discrete B- and T-lymphocyte subpopulations develop functional effector capabilities (i.e. immunoglobulin synthesis, cytotoxic activity, etc.). In addition, this interaction leads to further generation of cytokines (IL-2, IL-3, IL-4, IL-5, IL-10, GM-CSF, etc.) responsible for the further recruitment of peripheral blood lymphocytes, monocytes and polymorphonuclear leukocytes (PMN) and for the further activation of surrounding immunocompetent cells.

Growth factors such as PDGF, epidermal growth factor (EGF) and insulin-like growth factor (IGF-1) are produced by some cells which are activated in the airways of asthmatics (Barnes, 1994a). These factors stimulate fibrogenesis via their activation of

fibroblasts, and may also stimulate the proliferation and growth of airway smooth muscle cells (Hirst *et al.*, 1992).

Thus, inflammatory cells activated in asthma, release multiple cytokines that orchestrate and perpetuate the inflammatory responses. Cytokines can operate by autocrine, paracrine and endocrine mechanisms. They can inhibit or enhance their own production from their cell of origin or from adjacent cells, as well as influence the production of other cytokines. They are responsible for the recruitment of inflammatory cells from the circulation and activate structural cells (such as epithelial and endothelial cells) to favour this process. The network of interactions is complex.

PART C — ALVEOLAR MACROPHAGES

Introduction

In the normal lung, pulmonary macrophages are resident cells and are the most abundant nonparenchymal cell (Hunningshake *et al.*, 1979). They provide the first line of defense to inhaled particulate matter and microorganisms in the lower airways through their capacity to scavenge particulates, kill microorganisms, function as accessory cells in immune responses, recruit and activate other cells and maintain and repair the lung parenchyma (reviewed in Crystal, 1991). Pulmonary macrophages are grouped into two general populations; those that are a lavagable population, thus referred to as alveolar macrophages (AM), and those that are found in the interstitial spaces of the lung parenchyma, thus called interstitial macrophages (IM) (Lehnert *et al.*, 1985). Thus, anatomically the AM rests on the surface interface of the lung (i.e. in the alveolar space) and is likely the first and predominant cell type exposed to inhaled antigen.

Secretory Products and Surface Receptors

Macrophages can produce a wide range of secretory products, which include bioactive lipids (TXA_2, LTB_4, 5-HETE, PGE_2, PGD_2, PAF), oxygen metabolites, enzymes (lysozyme, elastase, proteinases and antiproteinases), and a large number of cytokines (IL-1α and β, IL-6, IL-8, IL-10, IL-12, TNFα, IFNα and γ, TGFβ, monocyte chemoattractant protein-1 and 3 (MCP-1 and MCP-3), macrophage migration inhibitory factor (MIF), colony stimulating factors (CSF), macrophage inflammatory protein-1 and 2 (MIP-1, MIP-2)) (Cavaillon, 1994; Kay, 1991; Sibelle, 1990). AM can interact with their environment through the binding of molecules on their cell surface. They possess receptors for bioactive lipids (LTB_4), histamine, IL-2, β-agonists, IgG, IgA, IgE, complement factors (C3, C5a), glucocorticoids, mannose/fructose, etc. (Sibelle, 1990; Crystal, 1991). These mediators all have biological activities that may contribute to airway reactions.

Macrophages in Asthma

Macrophages isolated from human BAL fluid are a heterologous population and can be isolated phenotypically and functionally into subsets (Spiteri *et al.*, 1992; Poulter *et al.*, 1986; Brain, 1988). Using monoclonal antibodies, lung macrophages have been classified as phagocytes, antigen presenting cells or suppressor cells (Poulter *et al.*, 1986). In bronchial biopsy and lavage from patients with asthma, the number of recovered macrophages has been shown to remain the same (reviewed in Arm and Lee, 1992) or to increase (Metzger *et al.*, 1987; Poston *et al.*, 1992). Studies have shown that AM recovered from asthmatic subjects do differ functionally and metabolically from those obtained from healthy subjects, and have been described as either "activated" or "postactivated". Goddard *et al.* (1982), showed that AM from asthmatics were less able to phagocytose zymosan particles and were less viable that normal AM. Also, AM from

atopic subjects released increased amounts of PAF when stimulated with specific allergen (Arnoux *et al.*, 1982; Arnoux *et al.*, 1987; Stenton *et al.*, 1990), and increased releaseability of superoxide anion and hydrogen peroxide (both pro-inflammatory initiators) that correlated with the severity of asthma and the degree of eosinophilia in the BAL fluid (Cluzel *et al.*, 1987). After antigen challenge, AM also produce TNFα and IL-6 consecutive to the LAR (Gosset *et al.*, 1991). Also, AM from asthmatics were less effective suppressor cells in that they function less well than normal AM in suppressing lectin-driven lymphocyte proliferative responses (Aubus *et al.*, 1984). The aberrant activation of macrophages, their phenotypical change and thus loss of regulatory function may account for their involvement in inflammatory diseases such as asthma.

Macrophages and IgG

Three classes of receptors for the Fc region of IgG have been identified in mice and humans, namely FcγRI, FcγRII and FcγRIII (Anderson *et al.*, 1988; Anderson, 1989; van de Winkel & Capel, 1993). Human FcγRI binds monomeric IgG (primarily of the IgG1 and IgG3 isotype) with high affinity (Ka = 10^8-10^9 M^{-1}), whereas FcγRII and FcγRIII bind only IgG containing immune complexes (FcγRII binds IgG1 and IgG3 and FcγRIII binds primarily IgG3) at lower affinities. (< 10^7 M^{-1}) (Fridman *et al.*, 1992, Rossman *et al.*, 1986; Anderson, 1989). In guinea-pigs, two distinct types of peritoneal macrophage FcγR have been characterized on the basis of their IgG subclass specificities. One is specific for both guinea-pig IgG1 and IgG2 ($Fc\gamma_{1/2}R$) and the other binds IgG2 alone ($Fc\gamma_2R$). These receptors resemble human and murine FcγRII and FcγRIII receptors, respectively, in terms of homology, their differential sensitivity to proteases, and their cellular distribution (Sugiyama *et al.*, 1981; Shimamura *et al.*, 1986; Shimamura *et al.*, 1987; Fukuchi *et al.*, 1992; Yamashita *et al.*, 1993). The affinity constants determined by these same investigators are recorded to be 1.7 x 10^8 M^{-1} for the

binding of OA complexed with IgG2, to the Fcγ_2R and 1.4 x 10^8 M^{-1} for the Fc$\gamma_{1/2}$R; the number of receptors for Fcγ_2R and Fc$\gamma_{1/2}$R are reported to be 1 x 10^5/cell and 2 x 10^5/cell, respectively (Nakamura *et al.*, 1988). Others have reported the association constant for the binding of monomeric IgG1 to guinea-pig peritoneal macrophages as 0.61 ± 0.22 x 10^6 M^{-1}, and IgG2 as 1.44 ± 0.16 x 10^6 M^{-1}, and the number of IgG1 and IgG2 receptor sites as 1.3 ± 0.2 x 10^6/cell and 2.65 ± 0.45 x 10^6/cell, respectively (Leslie & Cohen, 1976).

The Fc portion of the IgG molecules binds to the Fcγ glycoprotein receptor and the biological consequences that ensue include phagocytosis of IgG coated particles such as bacteria, the endocytosis of small IgG immune complexes, the secretion of inflammatory mediators such as superoxide, LTs, PGs and enzymes, the cell-mediated killing of target cells coated with IgG (i.e. ADCC), and modulation of the immune response by lymphocytes. Thus, Fcγ receptors and the cells that possess them, play an important role in ADCC, phagocytosis and regulation of immune responses (for reviews see Fridman *et al.*, 1992, Rossman *et al.*, 1986; Anderson, 1989; van de Winkel & Capel, 1993).

Table 1.2 summarizes general characteristics of human and guinea-pig Fcγ receptors (information collected from Anderson, 1989; Fridman *et al.*, 1992; van de Winkle & Capel, 1993; Sugiyama *et al.*, 1981; Shimamura *et al.*, 1986; Shimamura *et al.*, 1987; Fukuchi *et al.*, 1992; Yamashita *et al.*, 1993 and Nakamura *et al.*, 1988).

IgG and Allergy

IgE activation of mast cells and their subsequent degranulation and release of mediators has been viewed as the triggering event to allergic manifestations such as asthma. Human and murine mast cells also possess low affinity receptors for the Fc

TABLE 1.2

FcγR Class	MW(kDa)	Isoforms	Isotype Specificity	Cellular Distribution	Function
hFcγRI (CD64)	72	Ia, Ib_2, sIb_1, sIc	monomeric, high affinity 1=3>4>>>2	monocytes macrophages (neutrophils) (eosinophils)	phagocytosis oxidative burst ADCC, TNFα release
hFcγRII (CD32)	40	IIa, $sIIa_2$, IIc, IIb, IIb_2, IIb_3	complexed, moderate affinity IIa 3>1=2>>>4 IIb_1 3≥1>4>>2	monocytes macrophages neutrophils basophils eosinophils (B cells)	phagocytosis oxidative burst ADCC, TNFα release (regulation of Ig production)
hFcγRIII (CD16)	50-70	IIIaα, sIIIa, IIIb, sIIIb	complexed, moderate affinity 1=3>>>2,4	monocytes macrophages NK cells T cells (neutrophils)	(IIIa) phagocytosis oxidative burst ADCC, TNFα release clearance of immune complexes
GP $Fc_{\gamma 2}R$	52	?	complexed, monomeric? 2	macrophages ?	(phagocytosis) PI turnover Ca^{++} mobilization oxidative burst AA release
GP $Fc_{\gamma 1/\gamma 2}R$	55	b_1 = hFcγRIIb_2 b_2, b_3 } = hFcγ RIIb_1	complexed, monomeric? 1=2	macrophages PMN B cells ?	phagocytosis (oxidative burst) (AA release)

General characteristics of human and guinea-pig Fcγ receptors.

portion of IgG (Tigelaar *et al.*, 1971; Warner & Ovary, 1972), and IgG antibodies have been shown to activate mast cells (Ovary *et al.*, 1970; Prouvost-Danon, 1966; Oettgen *et al.*, 1994; Katz *et al.*, 1992). In contrast to early studies (Yamauchi *et al.*, 1986), it is now clear that IgG1 and IgG2 are anaphylactic shock antibodies in the guinea-pig (Desquand *et al.*, 1990). Guinea-pig mast cells and alveolar macrophages can be passively sensitized, *in vitro*, with antigen specific (ovalbumin (OA)) IgG1 or IgG2, and then activated to secrete histamine (by mast cells) and TXA_2 (by macrophages) upon OA challenge (Al-Laith *et al.*, 1993). In addition, antigen (OA) induces histamine and LTB_4/LTD_4 release by lung tissue extracted from guinea-pigs that were passively sensitized *in vivo*, with purified antigen specific (OA) IgG1 antibody (Cheng *et al.*, 1990). Anaphylactic histamine release has also been described from lung and tracheal tissues extracted from actively sensitized guinea-pigs (sensitized to respond with IgG antibodies) then challenged *in vitro* with antigen (Van Amsterdam *et al.*, 1990). As well, purified anti-ovalbumin IgG used for passive sensitization of guinea-pigs prior to OA inhalation challenge produced immediate- but not late-onset airway constrictive responses and pulmonary eosinophilia measured at 24 h post inhalation (Griffiths-Johnson *et al.*, 1993; Cheng *et al.*, 1993).

In a series of elegant transfection studies (using mast cell cell lines) designed to analyze the capacity of the FcγR to transduce such diverse signals as those leading to immediate (assessed by serotonin release) and synthesized (assessed by cytokine TNFα release) mediator release, it has been shown that both murine FcγRII, RIII and FcεRI associate with the same intracytoplasmic γ-homodimers, thus probably all using the same transduction pathway leading to the same biological activities (i.e. serotonin release and TNFα production) (Daeron *et al.*, 1992; Latour *et al.*, 1992; Fridman *et al.*, 1992). Recent studies from this group has demonstrated that IgE-induced release of serotonin (an example of an early allergic mediator) and TNFα (a cytokine probably involved in the

late phase reaction) can be inhibited by cross-linking of FcεRI to low affinity receptors for IgG (FcγRII) by the same multivalent ligand (Daeron *et al.*, 1995). This inhibition was dependent on intact intracellular domains of FcγRII, was reversible and was effective in mast cells previously sensitized with IgE. These extensive *in vitro* studies provide a structural role for IgG and Fcγ receptors in allergy.

Direct human studies addressing cytophilic IgG as a regulatory element in anaphylaxis are limited. Some human sera contains IgG antibodies and is capable of sensitizing monkey skin mast cells (Parish *et al.*, 1970). In allergic patients, allergen-specific IgG antibodies are frequently found in addition to allergen-specific IgE antibodies (Van der Zee & Aalberse, 1991; Soliman *et al.*, 1986; Kitani *et al.*, 1985). More recently, allergen-specific IgG1 and IgG3 (and not IgG4) interactions with FcγRII have shown to induce allergen-dependent degranulation of eosinophils collected from asthmatic patients (Kaneko *et al.*, 1995). Also, basophils collected from 12 atopic and 15 non-atopic patients, release histamine and LTC_4 when challenged with an antibody directed against the Fab (but not the Fc) region of the IgG4 molecule (Jimeno *et al.*, 1992), thus implicating a possible anaphylactic role for this immunoglobulin. Conversely, IgG antibodies induced by hyposensitization therapy are considered to act as allergen blocking antibodies by competitively inhibiting the binding of allergen to cell bound IgG. However, in asthma there is no significant correlation between the level of "blocking" antibody and relief of symptoms (Leynadier *et al*, 1991).

Together, these studies suggest the involvement of antigen-specific IgG, antigen and the various effector cells possessing Fcγ receptors, in Type I allergic responses in the airways. The following discussion outlines the involvement of TNFα in asthma and provides further rationale for assessing cytophilic IgG/FcγR-induced TNFα secretion in the context of airway disease.

PART D — TUMOR NECROSIS FACTOR ALPHA (TNFα)

Introduction

TNFα (also called cachectin), is a 17 kDa protein produced in large amounts by macrophages and in smaller amounts by lymphocytes, mast cells, basophils, eosinophils, natural killer (NK) cells, and B cells (Tracey, 1994; Beyaert & Fiers, 1994; Fiers, 1991; Aggarwal, 1992). The secreted protein is in the form of a compact trimer of three identical chains, that is glycosylated in the mouse but not in humans (Trinchieri, 1991; Van Ostade *et al.*, 1994). At the amino acid level, human TNFα has 79% homology with murine TNFα and 87% at the mRNA level (Pennica *et al.*, 1985). Guinea-pig TNFα (18 kDa) has been purified from guinea-pig peritoneal macrophages. Twenty-one residues in the NH_2-terminal of the protein were sequenced and shown to have 76 or 71% homology with mouse or human TNFα, respectively (Tamatani *et al.*, 1989). Also, rabbit antibodies to murine TNFα can be used to detect the guinea-pig cytokine (Ruppel-Kerr *et al.*, 1992). Macrophages, in addition to secreting the 17-kDa form of TNF can be induced to express a 26 kDa membrane bound form of TNF that is thought to also be biologically active in mediating macrophage-killing of TNF-sensitive target cells (Luettig *et al.*, 1989; Decker *et al.*, 1987; Kriegler *et al.*, 1988; Hofsli *et al.*, 1989). Cleavage of this 26 kDa membrane-bound form of TNFα by serine proteases is thought to also result in the production of the 17 kDa biologically active form of TNFα (Scuderi 1989). Human lymphotoxin (LT, TNFβ) shares 28% homology with human TNFα at the amino acid level and 46% at the nucleotide level (Gray *et al.*, 1984). TNFβ and TNFα share similar biological activities and interact with the same receptors, however TNFβ is primarily secreted from T lymphocytes (Trinchieri, 1991; Aggarwal, 1992).

Receptors for TNFα

Receptors for TNFα are widely distributed throughout most cells and tissues. Two known receptors exist, TNFα RI or TNFα Rp55 (apparent molecular mass of 55-60 kDa) and TNFα RII or TNFα Rp75 (apparent molecular mass of 75-80 kDa) (Camussi *et al.*, 1991; Smith & Baglioni, 1992). The dissociation constants for ligand with both types of receptors are reported to be in the picomolar range (Tracey, 1994; Aggarwal, 1992). A soluble form of the TNFα receptor (TNF-binding protein) has been detected in human urine and may be a soluble proteolysis product of the TNF receptor (Engelman *et al.*, 1989; Seckinger *et al.*, 1989). The effects of TNFα are transduced by the clustering of TNF receptors with the trimeric TNFα protein (Fiers, 1991). Binding and /or internalization of the complex activates PLA_2, resulting in the production of AA. As well, signaling occurs via specific C-type phospholipases, protein kinases and sphingomyelin (Schutze *et al.*, 1992; Pfizenmaier *et al.*, 1992).

Biological Effects of TNFα

The biological effects of TNFα are diverse. These may be beneficial or detrimental depending on the relative concentration of TNFα, as well as duration of cell exposure and presence of other mediators (Camussi *et al.*, 1991; Schutze *et al.*, 1992). Among many, *in vitro* biological effects of TNFα include apoptotic or necrotic cell lysis (Laster *et al.*, 1988), fibroblast proliferation (Sugarman *et al.*, 1985; Vilcek *et al.*, 1986), induction of procoagulant factor, leukocyte adhesion molecule (ELAM-1), membrane bound IL-1 and class I HLA on endothelial cell membranes, and secretion of IL-6, GM-CSF and plasminogen activating factor by endothelial cells (reviewed in Fiers, 1991; Camussi *et al.*, 1991; Van Hinsberg *et al.*, 1988). *In vivo,* biological effects of TNFα include cachexia (Cerami & Beutler, 1988; Tracey & Cerami, 1989), shock (via initial exposure to endotoxin) (Waage *et al.*, 1987), and inflammation (mediated through

the stimulation of second messengers (i.e. PAF and other pro-inflammatory cytokines), and the inducement and/or upregulation of cellular adhesion molecules on vascular and epithelial tissues (thus enhancing PMN recruitment)) (reviewed in Camussi 1991; Tracey, 1994; Fiers, 1991). TNFα is a mediator of the cytocidal activity of NK cells and macrophages (Ortaldo *et al.*, 1986; Urban *et al.*, 1986).

Inducers and Suppressors of TNFα Production

In humans, the genes for TNFα are on chromosome 1, near the region containing the MHC complex (Nedwin *et al.*, 1985). The production and secretion of TNFα can be regulated at a number of points along its biosynthetic pathway. Manipulation of the regulatory processes that govern TNFα DNA transcription, TNFα-mRNA stability, TNFα-mRNA translation, post-translational protein modifications, placement of the 26 kDa protein into the cell membrane and its subsequent release by proteolysis, or the secretion of the 17 kDa protein, are all points of therapeutic intervention in certain disease states that may be associated with excessive production of TNFα, a mediator of the inflammatory response. "Unactivated" macrophages have intracellular pools of TNFα-mRNA (Beutler *et al.*, 1986). Mast cells store pre-formed TNFα in secretory granules (Gordon & Galli, 1990) that can be released upon FcεR cross-linking. Storage of TNFα protein has not been demonstrated in any other cell type.

Many mediators are known to induce or inhibit TNFα secretion/production from cells, although their mechanisms are not entirely understood. Table 1.5 summarizes known inducers and suppressors of TNFα production (information compiled from Spriggs & Deutsch, 1992; Koerner *et al.*, 1987; Cannistra *et al.*, 1987; Beutler *et al.*, 1986; Burchett *et al.*, 1988; Spriggs *et al.*, 1990; Trinchieri, 1992; Tracey, 1994; Giroir & Beutler, 1992; Verghese *et al.*, 1995; Prabhakar *et al.*, 1994; Endres *et al.*, 1994; Semmler *et al.*, 1993; Sinha *et al.*, 1995; Sekut *et al.*, 1995; Seckinger & Dayer, 1992;

TABLE 1.3

Regulation of TNFα Production

Inducers	Suppressors
Cytokines IFNγ, IL-1, GM-CSF Protein Kinase C Phorbol esters Fc Receptors IgG immune complexes IgE plus antigen Increases in cGMP LTB_4 Transcription factor activators LPS	Cytokines IL-10, IFNγ, TGFβ, TNFα Secretion Inhibitors (via G-protein in cell membrane) Botulinium toxin Activators of Adenylate Cyclase Forskolin Cholera toxin Other increases in cAMP PGE_2 PDE inhibitors (theophylline, pentoxyfylline, amrinone, rolipram mRNA destruction Thalidomide Inhibitors of cleavage at cell membrane Serine proteinase inhibitors Transcription factor inhibitors Glucocorticoids

Moreira *et al.*, 1993; Sampaio *et al.*, 1991; Kim *et al.*, 1993).

Bacterial lipopolysaccharides (LPS) are the most potent inducers of macrophage TNFα production presently known. LPS is able to regulate transcriptional and post-transcriptional events in TNFα production after binding to an LPS-binding protein on the surface of macrophages (Beutler *et al.*, 1986; Burchett *et al.*, 1988). TNFα secretion/production is also induced by phorbol esters, and appears to again involve both transcriptional and posttranscriptional events (Spriggs & Deutsch, 1992). In the U937 cell line (myelomonocytic leukemia cells), GM-CSF induced increases in TNFα-mRNA levels in 2 h (Cannistra *et al.*, 1987), and TNFα induces its own expression at both the RNA and protein levels in HL-60 cells (Spriggs *et al.*, 1990). Also, IL-1 induces monocyte cytotoxic activity which is neutralized by anti-TNF antibody (Philip & Epstein, 1986). IFN-γ is a potent enhancer of TNFα production to stimuli such as LPS (Spriggs & Deutsch, 1992; Koerner *et al.*, 1987). Other stimuli inducing TNFα production/secretion are free oxygen radicals, LTB_4, H-ETE, IL-2 and Fc receptor cross-linking (reviewed in Spriggs & Deutsch, 1992; Trinchieri, 1992; Tracey, 1994).

Numerous studies indicate that mediators inducing rises in c-AMP levels are capable of decreasing production of TNFα. These include forskolin & cholera toxin (both activators of adenyl cyclase and thus increasers of c-AMP), and phosphodiesterase inhibitors such as theophylline, pentoxyfylline, amrinone and rolipram (Giroir & Beutler, 1992; Verghese *et al.*, 1995; Prabhakar *et al.*, 1994; Endres *et al.*, 1994; Semmler *et al.*, 1993; Sinha *et al.*, 1995; Sekut *et al.*, 1995). This effect is associated with decreased accumulation of TNF-mRNA following TNF stimulation (Spriggs & Deutcsh, 1992; Verghese *et al.*, 1995; Giroir & Beutler, 1992). Inhibition of TNFα production also occurs via PGE_2. Kunkel *et al.* (1988) demonstrate that exogenous PGE_2 dose-dependently suppresses LPS-induced TNF mRNA accumulation. The

lipoxygenase product, LTB_4, is an enhancer of TNFα production. Thus AA metabolites appear to regulate TNFα gene transcription, probably via c-AMP as a second messenger (Seckinger & Dayer, 1992).

Glucocorticoids also regulate TNFα production (Beutler *et al.*, 1986; Bendrups *et al.*, 1993; Barber *et al.*, 1993; Kerner *et al.*, 1992). Dexamethasone markedly suppresses LPS-induced increases in TNFα mRNA at both transcriptional and posttranscriptional levels (Beutler *et al.*, 1986).

Another compound, thalidomide, has been demonstrated to inhibit monocyte derived TNFα production by enhancing TNFα-mRNA degradation (Moreira *et al.*, 1993; Sampaio *et al.*, 1991). Whether or not this involves c-AMP, remains to be determined.

Inhibition of the protease that cleaves the primary translation product of TNFα into the soluble 17 kDa bioactive protein, intracellularly or from the cell membrane, is a mechanism whereby TNFα could be regulated after its synthesis. On the cell membrane, this has been demonstrated to likely involve serine proteinases (Kim *et al*, 1993).

TNFα and Asthma

The AM is a cellular source for the production and secretion of the pro-inflammatory cytokine, TNFα. There is good evidence that TNFα and IL-1 (also secreted by AM), have a variety of actions that could play a role in the initiation of the inflammatory response and other changes in the airways that are seen in asthma. TNFα has been demonstrated to cause airway hyperreactivity in a rat model of asthma (Kips *et al.*, 1992), to be recovered from the BAL fluid of asthmatic patients (Gosset *et al.*, 1991; Cembrzynska *et al.*, 1993; Brodie *et al.*, 1992) and to be involved in the process of leukocyte recruitment during an inflammatory response (Kips *et al.*, 1992; Wegner *et al.*, 1990). In evaluating the effect of AM supernatants from challenged asthmatic patients

developing a late-asthmatic response, Lassalle *et al.* (1991) showed a significant increase in ICAM-1 and ELAM-1 (cellular adhesion molecules that facilitate PMN transmigration from the vasculature and into the lungs) expression on endothelial cells, and TNFα concentrations in the supernatant correlated with this effect. Wershil *et al.* (1991), concluded that TNFα contributed to mast cell-dependent recruitment of leukocytes during IgE-dependent cutaneous late phase reactions. Ohno *et al.* (1994), reported that TNFα was released from sensitized lung tissue following IgE receptor triggering and Gosset *et al.* (1992) demonstrated IgE-dependent upregulation of TNFα and IL-1 secretion from AM and peripheral blood monocytes collected from asthmatic patients. Most recently, TNFα has been shown to significantly increase the responsiveness of human bronchial tissue to electrical field stimulation, *in vitro* . Responses were not increased in sensitized tissue nor in the presence of exogenous acetylcholine. These results show that TNFα causes an increase in responsiveness of human bronchial tissue that likely occurs prejunctionally on the parasympathetic nerve pathway (Anticevich, *et al*, 1995).

PART E — HYPOTHESES AND THESIS OBJECTIVES

It is clear that cytokines and growth factors orchestrate and perpetuate the chronic inflammation of asthma. A cytokine of particular interest is TNFα. As described, TNFα appears in the BAL fluid of asthmatic patients (Brodie *et al.*, 1992), and causes bronchial hyperresponsiveness in *in vivo* studies conducted in rats and sheep (Kips, *et al.*, 1992; Wheeler *et al.*, 1990). Cellular sources for TNFα include mast cells and AM and upon allergen inhalation, these cells are initial secreters of many inflammatory mediators, including TNFα and IL-1. These cytokines are essential for the perpetuation of the inflammatory response. They are capable of recruiting and activating many other immunocompetent cells and also activate structural elements such as epithelium (reviewed in detail in previous sections).

A cell capable of producing TNFα is the macrophage. Anatomically, the AM rests on the surface interface of the lung and is likely the first and predominant immunocompetent cell type exposed to inhaled allergen. It is likely that AM are activated upon inhalation of allergen. In antibody-dependent cell mediated reactions, macrophages liberate TNFα as part of the cytocidal mechanism.

Human studies addressing cytophilic IgG as a regulatory element in anaphylaxis are limited. In allergic patients, allergen-specific IgG antibodies are frequently found in addition to allergen-specific IgE antibodies (Van der Zee & Aalberse, 1991; Soliman *et al.*, 1986; Kitani *et al.*, 1985) and more recently, allergen specific IgG1 and IgG3 interactions with FcγRII have shown to induce allergen-dependent degranulation of eosinophils collected from asthmatic patients (Kaneko *et al.*, 1995). Guinea-pig AM can be passively sensitized, *in vitro*, with antigen specific IgG1 or IgG2 and then activated to secrete TXA_2 upon OA challenge (Al-Laith *et al.*, 1993). The guinea-pig Fcγ receptors that bind both IgG1 and IgG2, are homologous to human FcγRII receptors. Thus, the guinea-pig provides a relevent model for studying these processes. In terms of airway disease, guinea-pigs have been reported to exhibit early- and late-onset airway obstruction, bronchial eosinophilia and increased airway reactivity following exposure to antigen following sensitization (Kallos & Kallos, 1984). They can be sensitized to allergen more easily than other species and their lungs are also well innervated with neuropeptide-containing c-afferent fibers. Thus, for our studies, we chose the ovalbumin-sensitized guinea-pig model of asthma. Guinea-pigs are passively sensitized to ovalbumin, followed 24 h later by inhalation of ovalbumin aerosols. They then develop the above classical symptoms of human asthma. Using this model, we hypothesized that:

1. TNFα is an *early* mediator released from cells and thus into the BAL fluid post antigen challenge in our guinea-pig model of asthma.

2. A cell capable of enhanced TNFα production post antigen challenge, *in vivo*, is the AM.

3. Tachykinin neuropeptides may partly regulate TNFα production in our *in vivo* model, and *in vitro* from AM. (SP has been shown to induce the release of IL-1, TNFα and IL-6 from human blood monocytes (Lotz *et al.*, 1988)).

4. Thalidomide, a compound known to inhibit TNFα production from monocytes and macrophages, may reduce agonist-induced airways' hyperresponsiveness in our *in vivo* antigen-based guinea-pig model of airway hyperreactivity.

5. The activation of AM by allergen to produce TNFα, may involve cross-linking of Fcγ receptors via cell bound antigen specific IgG, plus antigen.

Using a guinea-pig model of airway hyperreactivity, the goals of our research were to:

1. Evaluate TNFα production into BAL fluid in our passively immunized then challenged guinea-pig model of asthma.

2. Investigate the *in vitro* production of the cytokines, TNFα, IL-1 and IL-6 from AM that were collected after *in vivo* antigen challenge.

3. Investigate the production of TNFα into lavage fluid and from cultured AM from antigen-challenged guinea-pigs that are pre-treated with capsaicin.

4. Investigate *in vitro*, the inhibition of LPS-induced TNFα production by thalidiomide (Thalidomide inhibits monocyte derived TNFα production by enhancing TNFα-mRNA degradation (Moreira *et al.*, 1993)).

5. Assess the ability of thalidomide (infused by osmotic pump prior to and during antigen sensitization and challenge) to alter airway mechanics (resistance and elastance) in ventilated anesthetized guinea-pigs receiving intravenous histamine and LTC_4.

6. To produce a guinea-pig/murine heterohybridoma that secretes ovalbumin specific monoclonal-antibody of guinea-pig IgG isotype, in order to investigate IgG/FcγR regulation of TNFα production by AM, *in vitro* and to refine the passive sensitization models by replacing the pAb with a mAb.

7. If (6) proves unsuccessful, to purify antigen-specific polyclonal IgG from the serum of actively immunized guinea-pigs.

8. To perform *in vitro* studies assessing antigen activation of AM after incubation with ovalbumin specific mAb (i.e. from (6) above) or ovalbumin specific pAb (i.e. from (7) above). Parallel studies would address TNFα production due to cross-linking of bound whole IgG with anti-IgG antibodies (bought commercially).

CHAPTER 2

RELEASE OF TUMOR NECROSIS FACTOR α INTO BRONCHIAL ALVEOLAR LAVAGE FLUID FOLLOWING ANTIGEN CHALLENGE IN PASSIVELY SENSITIZED GUINEA-PIGS

SUMMARY

These studies were undertaken to determine whether TNFα, IL-1 and IL-6 could be detected in guinea-pig BAL fluid immediately after antigen challenge. Guinea-pigs were passively sensitized with ovalbumin (OA) anti-serum, or control guinea-pig serum. Twenty-four hours later, they inhaled aerosols of OA, saline or LPS. Their lungs were lavaged at 30, 60, 90 or 120 min post-inhalations. The BAL fluids were centrifuged and frozen until assayed for cytokines. Results indicate that no TNFα could be detected in the BAL fluid unless aprotinin was present in the lavaging solution. BAL fluid from OA-sensitized and control animals that had inhaled LPS (as a positive control), contained high levels of TNFα that peaked at 90 min. BAL fluid from OA-sensitized animals that inhaled OA aerosols contained no detectable TNFα at 30 min, but it was found in increasing amounts at 60, 90 and 120 min; TNFα was not detected in the fluid from any of the animals that inhaled saline. Neither IL-1 or IL-6 could be measured, as BAL fluids were toxic to the cells used in the bioassays. Thus, TNFα is detectable in the BAL when lavage is performed 60 min following antigen challenge (by inhalation) of passively sensitized guinea-pigs. As the interval between challenge and lavage increases, so do amounts of TNFα detectable in the BAL.

INTRODUCTION

Cytokine-mediated interactions among macrophages, lymphocytes and eosinophils appear to be involved in the pathogenesis of the eosinophilia and airways' inflammation that characterize asthma (Brown *et al.*, 1990; Camussi *et al.*, 1991; Schollmeier, 1991; Semenzato, 1990). Thus, tumour necrosis factor α (TNFα) and granulocyte-macrophage colony-stimulating factor (GM-CSF), given parenterally, induce the accumulation of eosinophils in the airways of normal guinea-pigs (Kings *et*

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al., 1990). Also, TNFα is released from sensitized lung tissue following IgE receptor triggering (Ohno *et al.*, 1991) and it contributes to mast cell dependent recruitment of leukocytes during IgE dependent cutaneous late phase reactions (Wershil *et al.*, 1991). Alveolar macrophages isolated from bronchial alveolar lavage (BAL) fluid from allergen challenged patients undergoing a late asthmatic response secrete greater amounts of TNFα and interleukin-6 (IL-6) than macrophages isolated from BAL fluid of patients who develop no response or only an early response to allergen challenge (Gosset *et al.*, 1991). However, Gosset *et al.* (1991) were unable to detect TNFα in BAL fluid from asthmatic patients challenged with antigen inhalation. By contrast, Broide *et al.* (1992) showed that levels of several cytokines, including TNFα, IL-1 and IL-6, were significantly elevated in BAL fluid from patients with symptomatic asthma compared to asymptomatic asthmatic controls. We used guinea-pigs passively sensitized to ovalbumin (OA), and determined whether the cytokines TNFα, IL-1 and IL-6 could be detected in BAL fluid immediately after challenge with an aerosol of OA. Guinea-pigs passively sensitized to OA and to inert serum that inhaled LPS served as positive controls for TNFα release.

METHODS

Animals

Five groups of ten female Hartley strain guinea-pigs, SPF quality, weight range 320-400 g, obtained from Charles River, St. Constant, Quebec, were housed in laminar flow units (Bioclean, Hazleton, MD) on grids in cages suspended over trays of rock salt. They were fed normal guinea-pig chow supplemented with apples and allowed water *ad libitum*. They were monitored for at least one week after being shipped to ensure that they were in good health. They weighed 350-450 g at experiment.

Experiments

In each of the five groups of ten guinea-pigs, nine were passively sensitized to OA with guinea-pig hyperimmune serum (anti-OA antibody titre = 1:2500, by ELISA; 0.2 ml, i.p.), and one animal received control guinea-pig serum (0.2 ml, i.p.). One day later, all animals received mepyramine (0.5 mg/kg, i.p.) and 30 min later, the nine guinea-pigs that had received hyperimmune serum inhaled aerosols (Vix AcornTM nebulizer, compressed air at 10 psi) of: (A) OA (2% in 0.9% saline, up to 8 min, n = 4/9); (B) 0.9% saline (as for (A) n = 4/9); (C) LPS (E. coli 0111:B4, 150 ng/ml in PBS, 40 min, n = 1/9; and (D) the guinea-pig that had received control serum was treated as in (C). At 30, 60, 90 and 120 min after challenge with OA or LPS, the groups of ten animals were anesthetized (pentobarbital, 40-50 mg/kg, i.p.), their tracheas cannulated (PE240) and BAL performed.

Bronchial Alveolar Lavage

One group of guinea-pigs' lungs was lavaged with 2 x 5 ml PBS containing BSA (1%, to maintain viability of any cells recovered); the other four groups were lavaged with 2 x 5 ml PBS containing BSA and aprotinin (1000 KIU/ml, to inhibit protease activity. BAL fluids recovered were centrifuged (2000 rpm, 5 min) and the supernatant removed and immediately frozen at -20°C until assay.

Cytokine Measurements

All cytokine assays were performed in triplicate. Levels of TNFα were measured by the specific ability of this cytokine to exert cytotoxicity versus the L929 fibroblast cell line (Hogan & Vogel, 1991). Briefly, L929 cells (10^5) in complete medium [100 µl RPMI 1640, with 10% FBS, 1% penicillin-streptomycin, and 1 µg/ml actinomycin D (Sigma)] were incubated with aliquots of supernatants for 18 h at 37°C. Then,

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 25 µl; Sigma) was added to each well. After incubation (4 h), acidified isopropanol was added and optical densities read at 570 nm. The cytotoxic activity of all supernatants from AM was completely blocked by polyclonal rabbit anti-human-TNFα serum (5 µg/ml; Olympus - Corp., New York, New York); preimmune rabbit serum from the same source had no effect. After probit transformation, concentrations of TNFα were calculated from a standard curve established with human recombinant TNFα (hrTNF-α, Amgen).

Levels of IL-l were determined from the ability of supernatants to support the concanavalin A (Con A) -driven growth of D10.G4.1 cells (De Rochemonteix-Galve *et al.*, 1991; Muegge & Durum, 1991). Briefly, cells (4×10^5) were suspended in complete medium (100 µl) containing Con A (2.5 µg/ml, Sigma) with log 2 dilutions of supernatants. After incubation (72 h, 37°C), growth was assessed by the MTT procedure. Levels of IL-l-like activity were derived from a standard curve prepared with human rIL-l (Genzyme). An anti-human IL-1 monoclonal antibody (Genzyme) failed to block the growth-stimulating effects of the activity present in the supernatants (both BAL fluid and AM supernatants (see Chapter 3)). Thus, any IL-1 activity described in this thesis is actually "IL-1-like" activity.

Levels of IL-6 were determined as described (Aarden *et al.*, 1987). Briefly, B9 hybridoma cells (10^5) were incubated for 72 h in fortified RPMI 1640 medium in the presence of log 2 dilutions of supernatants. Growth stimulation was assessed by the MTT procedure. Levels of IL-6-like activity were derived from a standard curve prepared with human rIL-6 (British Biotechnology, Oxford, UK). Polyclonal rabbit anti-human-IL-6 serum (5 µg/ml) (Genzyme) did not prevent the growth-stimulating effects of the activity present in the supernatants (both BAL fluid and AM supernatants (see Chapter 3)). Thus, any IL-6 activity described in this thesis is actually an

"IL-6-like" activity.

Preparation of Guinea-Pig Sera

On day 0, guinea-pigs were given OA (20 mg/kg, i.p.); Twenty-one days later, they inhaled OA aerosols (2% in saline, mepyramine 0.5 mg/kg given 30 min beforehand, ip) for up to 8 min, on 8 consecutive days. On day 35, they were anesthetized (pentobarbital, 50 mg/kg, ip) and blood collected via cardiac puncture into plain glass tubes. Tubes were stored at 4°C overnight to allow the clot to form and retract. Serum was collected after centrifugation, pooled and stored at 4°C until use.

Estimation of Anti-OA-Antibody Titers as IgG

Serum was assayed for anti-OA-IgG antibodies using ELISA. Microtiter plates (Nunc immunosorbent plates, Maxisorp, Gibco, Burlington, Canada) were coated with OA (20 µg/ml, 75 µl) at 4°C for 48 h. After aspiration, nonspecific binding sites were blocked with undiluted skim milk at 22-24°C for 2 h. Plates were washed twice with Wash Solution (0.002 M imidazole buffered saline containing 0.02% Tween 20, Kirkegaard and Perry Laboratories Inc., Gaithersberg, Maryland) using an automated washer (Maxline model 4845-02, Molecular Devices, Menlo Park, California). Aliquots of immune or control sera were diluted with PBS (1 in 100), then log 2 dilutions of sera (50 µl) were added to the wells and incubated at 22-24°C for 1 h. After aspiration, rabbit anti-guinea pig IgG (50 µl, Sigma) was added to each well and plates were incubated (22-24°C, 1 h). After two washes, anti-rabbit IgG-HRPO (50 µl, Sigma) was added to each well and plates were incubated (22-24°C, 1 h). After three washes, ABTS/H_2O_2 (50 µl, Sigma) was added to each well and plates were incubated (22-24°C, 0.5 h). Optical densities were read (405 nm) using an automated plate reader and titers estimated (V Max model 04662; SOFTmax Software, Version 2.01d, Molecular Devices). Intra-

and interassay coefficients of variation ranged from 2 to 8% depending on dilution (data not shown). Sera were diluted to a titer of 2000 with PBS and stored at -20°C until used. Anti-OA-IgG antibodies were not detected in control sera. Anti-OA-IgE antibodies were estimated using the passive cutaneous anaphylaxis (PCA) method but could not be detected in immune or control sera (data not shown, see Chapter 8 for PCA methods).

Reagents and Chemicals

Actinomycin D, aprotinin, concanavalin A, 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolinium bromide (MTT), hen egg albumin (ovalbumin, Grade V) and LPS (*E. coli* 0111:B4) (Sigma, St Louis, MO), penicillin-streptomycin mixture and RPMI 1640 medium (Gibco, Grand Island, NY) FBS (PA Biologicals, Sydney, Australia), recombinant human TNFα and human recombinant IL-1, (Amersham Canada Ltd., Oakville, Ontario), rabbit anti-human TNFα and pre-immune serum (Olympus Corp., New York, NY), and human recombinant IL-6 (British Biotechnology, Oxford, UK).

Statistical Analyses

Data were expressed as mean ± S.E.M. Differences among and between the groups were examined using ANOVA. Differences were assumed significant at the 5% level.

RESULTS

TNFα Concentrations

In the absence of aprotinin, TNFα could not be detected in BAL fluids obtained at 60 min post-challenge whether guinea-pigs had inhaled OA saline or LPS (positive controls) aerosols. However, if animals' lungs were lavaged with fluid containing the

antiprotease, aprotinin (1000 KIU/ml), significant amounts of TNFα were detected (Figure 2.1). In the group challenged with OA aerosol, TNFα was not detectable in BAL fluid collected 30 min post-challenge, but increasing amounts of TNFα were detected at 60, 90 and 120 min post-challenge and correlated positively with the time BAL fluid was collected postchallenge. By contrast, TNFα was not detected in BAL fluid from animals challenged with saline. However, TNFα was detected in BAL fluid from both positive controls, (i.e the passively sensitized and control serum treated animals that had inhaled LPS aerosol) (Figure 2.1). The cytotoxicity of this TNFα was prevented if the BAL fluid, rabbit anti-human TNFα serum (5 μl) and the L929 cells were incubated together; rabbit pre-immune serum (5 μl) was without effect on the cytotoxicity. (BAL at 90 min: OA/OA: + preimmune serum, [TNFα] = 21 ± 8 U/ml; +anti-TNFα serum, [TNFα] = < 2.0 U/ml; OA/LPS: + preimmune serum, [TNFα] =71± 10 U/ml; + anti-TNFα serum, [TNFα] = < 2.0 U/ml).

IL-1 and IL-6 Concentrations

Neither IL-1 nor IL-6 could be detected in BAL fluid as the latter was toxic to the cell lines used to assay for the cytokines. Dialysis of the BAL fluids for 24 h failed to completely eliminate this toxicity.

Acute Effects of Inhalation of OA, Saline and LPS Aerosols in Guinea-Pigs

In all guinea-pigs that were passively sensitized to OA and inhaled OA aerosols, some signs of anaphylaxis were noted despite the presence of mepyramine. Animals were carefully observed and those that showed signs of respiratory distress were immediately removed from the exposure chamber to prevent progression to severe respiratory distress and death. Exposure times in the OA/OA groups ranged from 0.5 to

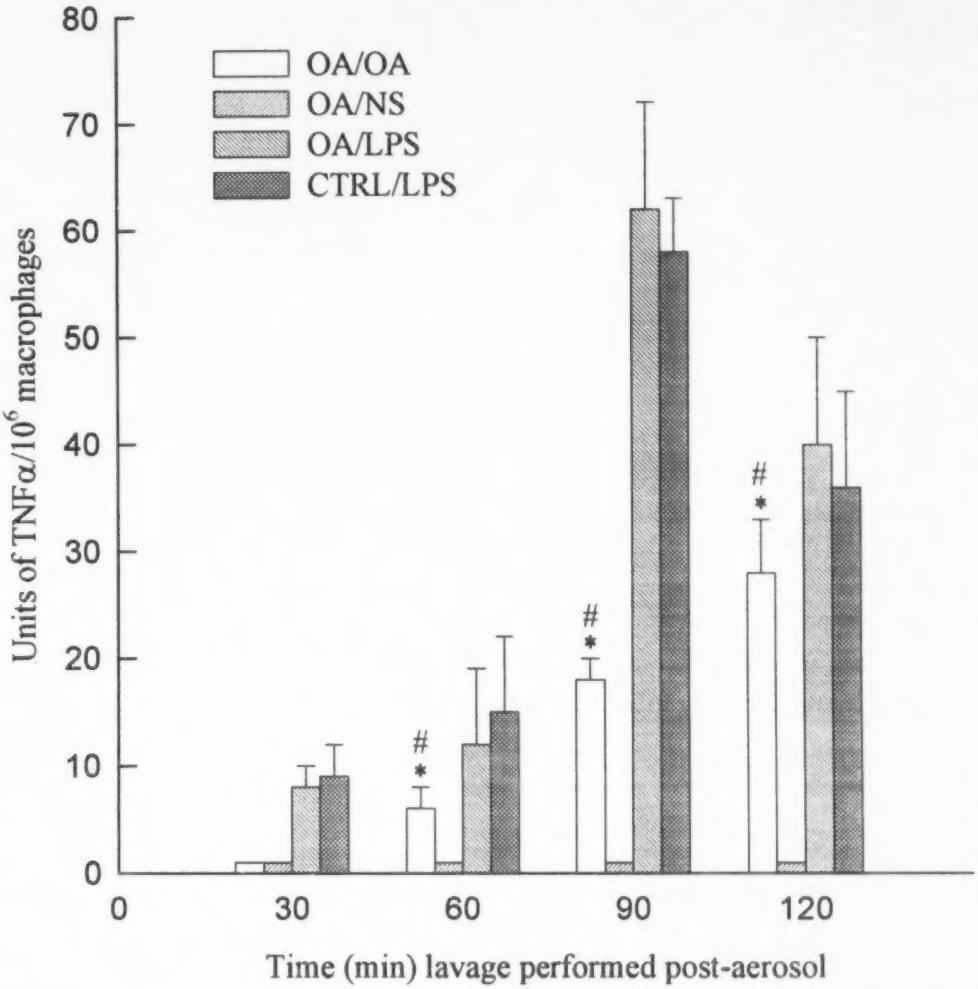

Figure 2.1. Amounts of TNFα (in units/10^6 AM) detected in BAL fluid (aprotinin, 1,000 KIU/ml present in lavaging solution). Guinea-pigs were divided into four groups. (A) Sensitized guinea-pigs given OA aerosol (OA/OA, n = 4); (B) sensitized guinea-pigs given saline aerosol (OA/NS, n = 4); (C) sensitized guinea-pigs given LPS aerosol (OA/LPS, n = 1); (D) control immunized guinea-pigs given LPS aerosol (CTRL/LPS, n = 1). 30, 60, 90 and 120 min post-aerosol, animals were anaesthetized and BAL performed. * Denotes significantly different from time 30 min ($p < 0.05$, ANOVA). # Denotes a significantly higher release in OA/OA group compared to OA/NS group ($p < 0.05$, ANOVA).

8 min. Mean exposure time was 4.4 ± 0.7 min (n = 16). Duration of exposure to OA aerosol did not correlate with the amount of TNFα detected in BAL fluid.

DISCUSSION

We report the detection of TNFα-like activity in BAL fluid obtained from guinea-pigs passively sensitized to OA and challenged with OA aerosols. TNFα-like activity could not be detected at 30 min post-challenge, but it was measurable (8-26 U/ml) in fluid obtained at 60, 90 and 120 min post challenge. As rabbit anti-human TNFα antibodies prevented the cytotoxicity of the lavage fluids, it is highly likely that it is TNFα. TNFα levels in BAL fluids from animals that had inhaled LPS appeared to peak about 90 min post-aerosol inhalation. It is worth noting that these guinea-pigs inhaled LPS aerosol for 40 min compared to OA aerosol for up to 8 min in the other group. Thus, as we have not defined the peak of TNFα secretion into BAL fluid for OA challenged animals, we cannot say whether 120 min represents the maximal concentration that may be achieved or whether its pattern of secretion follows that seen after LPS inhalation. The levels of TNFα detected in these experiments are similar to those found by others in BAL fluid from guinea-pigs after inhalation of LPS (De Rochemontieux-Galve *et al.*, 1991) or cotton dust (Ryan & Karol, 1991).

Unlike Broide *et al.* (1992) who measured 'resting' TNFα levels in BAL fluid from symptomatic and asymptomatic asthmatics, and like Gosset *et al.* (1991) we could not detect TNFα unless a protease inhibitor was present in the lavaging solution. This is noteworthy. All the passively sensitized guinea-pigs showed signs of anaphylaxis upon inhalation of OA aerosols, but the duration of exposure to the OA aerosol did not appear to influence the amount of TNFα detected in BAL fluid. Others (Caughey *et al.*, 1988; Miller *et al.*, 1983) have shown that antigen challenge of sensitized animals leads not

only to the release of a variety of mediators, but also to the release of proteases that could metabolize cytokines such as TNFα. Thus, as BAL was performed 30-120 min after challenge, lack of aprotinin in the lavaging solution could have allowed metabolism of any TNFα released and thus prevented its detection. This finding emphasizes the importance of adding antiproteases to lavaging solutions to help prevent breakdown of cytokines during and after BAL.

Others (Ohno *et al.*, 1991; Wershil *et al.*, 1991; Borish *et al.*, 1991) have reported that IgE dependent reactions result in TNFα formation and secretion. In our experiments, the sensitizing antibody is most likely to be a subtype of IgG, probably IgGl or IgG2, as passive cutaneous anaphylaxis tests (data not shown) revealed no IgE in the serum used to passively sensitize animals. These findings show that passive sensitization with anti-OA antibodies of the IgG class followed by antigen challenge can induce directly or indirectly the production of TNFα. The production of TNFα is not unique to a particular cell type, but a major source of TNFα is macrophages (Camussi *et al.*, 1991). Gosset *et al.* (1991) and Borish *et al.* (1991) showed that alveolar macrophages collected from antigen challenged asthmatics undergoing a late reaction generated significantly more TNFα than similar patients who underwent only an early reaction or who had experienced no reaction. We speculate that, in our experiments in guinea-pigs, most of the TNFα that is detectable is secreted from alveolar macrophages. Neither IL-1 nor IL-6 could be detected as the lavage fluid was toxic to the cell types used in the bioassay of these cytokines. Preliminary experiments (data not shown) showed that ELISAs for murine IL-1 and IL-6 could not demonstrate the presence of these cytokines in BAL fluid. Thus, whether these cytokines are present in physiologically significant amounts in BAL fluid remains to be determined.

The physiological significance of TNFα release following passive sensitization

and antigen challenge is unclear. Our data suggest that the release of proteases and other mast cell associated mediators during anaphylaxis may act to reduce TNFα's pro-inflammatory effects. Gosset *et al.* (1992) reported that human alveolar macrophages from asthmatics have an enhanced capacity to produce TNFα, compared with cells from normal control subjects. Thus, TNFα may have a greater role in the chronic inflammatory processes in asthma than was previously supposed.

We conclude that TNFα is released in passively sensitized guinea-pigs' lungs soon after antigen inhalation. A protease inhibitor must be present in the lavaging solution to enable TNFα's detection.

CHAPTER 3

RELEASE OF MONOKINES BY PULMONARY MACROPHAGES FOLLOWING ANTIGEN CHALLENGE IN SENSITIZED GUINEA-PIGS

SUMMARY

Thus, TNFα is detectable in the BAL fluid as early as 60 min following antigen challenge (by aerosol) of passively sensitized guinea-pigs. A known cell source for TNFα is the AM. This study examines TNFα, IL-1 and IL-6 production from AM recovered from the BAL fluid of guinea-pigs passively sensitized to OA, then challenged with OA aerosol 24 h later. AM were collected at 30, 60, 90 and 120 min post antigen or LPS aerosol, then incubated for a further 18 h. Cell supernatants were assayed for cytokines. Results indicate that antigen challenge causes AM to produce increased amounts of IL-6 and TNFα (spontaneously and in response to zymosan) compared to AM from control animals aerosoled with saline. IL-6 and TNFα were detectable in AM supernatants when lavage was performed at 30, 60, 90 or 120 min post antigen aerosol. AM released IL-1 at constant levels at all lavage time periods examined, both in challenged and unchallenged groups. LPS challenge (positive control) resulted in production of large amounts of IL-1, TNFα and IL-6 by AM, both spontaneously and in response to zymosan. These findings show that antigen challenge results in activation of AM obtained by BAL. The findings are compatible with the concept that bronchoalveolar macrophages participate in the development of inflammatory processes associated with local antigen challenge.

INTRODUCTION

Immediate hypersensitivity reactions in the lungs have been the subject of considerable investigation, much of which has focused on the impact of mast cells and their products in mediating physiologic events such as bronchospasm (Wasserman, 1979) and cellular events such as the influx of inflammatory cells (Wasserman, 1979; 1983). Proinflammatory mediators such as histamine, thromboxane A_2 (TXA_2), leukotrienes, and platelet-activating factor (PAF) are involved in the development of the sequence of events that follow anaphylactic challenge (Shalit *et al.*, 1989). Antagonism

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of the receptor mediated effects of these substances in animal models of asthma has demonstrated their involvement in the development of airway hyperresponsiveness and cell recruitment (Kreutner *et al.*, 1989; Ishida *et al.*, 1990). These substances may also interact with, and modulate the activity of leukocytes (Fuller *et al.*, 1986).

Alveolar macrophages (AM) are present in large numbers in the bronchoalveolar spaces and are likely to be exposed to inhaled antigen. Following antigen challenge, AM secrete lysosomal enzymes, superoxide anion, and cytokines (Joseph *et al.*, 1983; Rankin *et al.*, 1984). The activation status of AM from asthmatic patients is augmented (Cluzel *et al.*, 1987) and monokine release by AM from allergic patients is increased after the development of a late asthmatic reaction (Gosset *et al.*, 1991). In a passively sensitized guinea pig model of asthma (Ladenous & Biggs, 1989), it was shown that TNFα can be detected in bronchoalveolar lavage (BAL) fluid from between 0.5-2 h after antigen challenge (Kelly *et al.*, 1992). In these experiments, we used this model to determine whether AM secrete monokines, such as TNFα, during the early events that follow antigenic challenge in the lungs and result in accumulation of T lymphocytes and eosinophils (Frew & Kay, 1988). Guinea pigs that inhaled lipopolysaccharide (LPS) served as positive controls for these experiments (Hogan & Vogel, 1988).

MATERIALS AND METHODS

Preparation of Guinea-Pig Sera

As described in Chapter 2, page 48.

Estimation of Anti-OA-Antibody Titers as IgG

As described in Chapter 2, page 49.

Sensitization and Challenge

Guinea pigs (see above) were given anti-OA-IgG-antibody-containing (immune) serum (0.4 ml, intraperitoneally), or saline (0.4 ml/kg, intraperitoneally). Twenty-four hours later, they were divided into five groups and subjected to aerosol challenges as follows: group A (N = 8, OA/OA) received immune serum and inhaled OA aerosol (2% in saline) for up to 8 min; group B (N = 8, OA/NS) received immune serum and inhaled saline aerosol for 8 min; group C (N = 2, NS/OA) received saline (intraperitoneally) and inhaled OA aerosol (2% in saline) for 8 min; group D (N = 2, OA/LPS) received immune serum and inhaled LPS (150 ng/kg, *E. coli* 0111:B4, Sigma) for 8 min; and group E (N = 2, CTRL/LPS) received saline (intraperitoneally) and inhaled LPS (150 ng/kg) for 8 min. Aerosol challenges were performed as described above. All animals received pyrilamine (0.5 mg/kg) intraperitoneally, 30 min before challenge. Thus, 22 guinea pigs were used for the collection of AM, four times after aerosol challenge (30, 60, 90, and 120 min) for a total of 88 animals. In addition, four animals that had received no treatments (group F) were used as an additional control.

Collection of Alveolar Macrophages (AM)

Guinea pigs were anesthetized with pentobarbitol (45-50 mg/kg, intraperitoneally; 5 mg/kg increments, intravenously, if required). Their tracheas were exposed by a midline incision and cannulated (PE240). The lungs were lavaged with 2 x 5 ml sterile PBS (calcium- and magnesium-free phosphate-buffered saline, pH 7.4) containing bovine serum albumin (1%, Sigma) and aprotinin (1000 KIU/ml, Sigma); about 8.0 ml of fluid was recovered from each animal. Fluids were centrifuged (2000 rpm, 5 min) and the cell pellet resuspended in RPMI 1640 medium (5 ml) containing penicillin (5 units/ml) and streptomycin (50 mg/ml) (Penicillin-streptomycin Solution, Sigma) and

fetal bovine serum (FBS, 5%, Sigma). Tubes were packed in storage containers at 4°C and shipped overnight from Edmonton to Sherbrooke.

Isolation and Stimulation of AM

Upon arrival, total (trypan blue) and differential (Diff Quick, Baxter, McGaw Park, Illinois) cell counts were performed. Cell viability was >97% as assessed by trypan blue exclusion. Cells were plated at 1.5 x 10^6/ml in each well of plastic 24-well plates (Corning Glass Works, Corning, New York) and incubated for 2 h at 37°C. Nonadherent cells were removed by vigorous washings with warm medium. Cells were >97% AM (Wright-Giemsa) after this procedure. Then, cells were left untreated, or stimulated with zymosan (1 mg/ ml), and incubated for 18 h at 37°C. Supernatants were collected, filtered and frozen (-80°C) until assayed for monokines. To standardize data, adherent cells (AM) were dislodged from each well with cold EDTA solution (1%) and aliquots taken and cells counted as above. This allowed expression of amounts of cytokines as units per 10^6 AM. Endotoxin contamination in our media was < 50 pg/ml (E-Toxate test, Sigma). All glassware was washed in E-Toxa-clean (Sigma) and baked overnight to destroy endotoxin.

Cytokine Measurements

As described in Chapter 2, page 47.

Statistical Analyses

Differences between groups were compared by Students' t test. Differences among groups were examined using ANOVA (SPSS-X) and Student-Newman-Keuls' tests. Significance was assumed at the 5% level.

RESULTS

Numbers and Types of Cells Collected

There were no statistically significant differences among the numbers of cells (range: 2.0-3.0 x 10^6) collected by lavage in groups A-E and the untreated controls (group F). Similar findings were obtained when adherent cells were counted. The types of cells, as determined by differential staining, were similar among groups A-E. The majority of the cells (>80%) appeared to be macrophages; neutrophils and eosinophils comprised 2-5%. No basophils were noted. A few ciliated epithelial cells were noted in cells collected from animals in group A (OA/OA). Adherent cells appeared to be almost exclusively alveolar macrophages (AM).

Release of TNFα by Adherent Cells

Findings are summarized in Figures 3.1 and 3.2. AM from guinea-pigs in groups D (OA/LPS) and E (CTRL/LPS) that had inhaled aerosols of LPS released TNFα (50-70 units/10^6 AM) spontaneously at all collection times. AM from these groups released similar amounts of TNFα in the absence and presence of zymosan (1 mg/ml) ($P > 0.05$). AM collected at all times post challenge from guinea pigs in group B (OA/NS) (passively sensitized to OA, saline aerosol challenge) failed to release detectable amounts of TNFα (<0.2 units/ml). After stimulation with zymosan, AM in this group collected at all times post challenge released similar amounts of TNFα (about 20 units/10^6 AM). By contrast, AM collected 30 min post challenge from guinea pigs in group A (OA/OA) (passively sensitized to OA, OA aerosol challenged) released detectable amounts of TNFα (4 ± 2 units/10^6 AM); AM collected at 60, 90, and 120 min post challenge released significantly greater amounts (15-20 units/10^6 AM, $P < 0.05$) than this. Zymosan stimulation markedly increased the production of TNFα (4- to

12-fold) in cells from group A ($P < 0.0001$ at all times examined).

Production of IL-1

Figures 3.3 and 3.4 summarizes the data on IL-1 release. Because an anti-human IL-1 monoclonal antibody (Genzyme) failed to block the growth-stimulating effects of the activity present in the supernatants, any IL-1 activity described in this thesis is "IL-1-like" activity. Spontaneous IL-1 secretion was similar for all groups at 30, 60, and 90 min post aerosol. At 120 min post aerosol, AM from the LPS-exposed groups released more IL-1 than any other groups, at all times examined. Zymosan stimulation significantly increased IL-1 release from AM from all groups and at all times examined except for the LPS-exposed groups at time 120 min ($P < 0.004$).

Production of IL-6

Results obtained with IL-6 are summarized in Figures 3.5 and 3.6. Because an anti-human IL-6 monoclonal antibody (Genzyme) failed to block the growth-stimulating effects of the activity present in the supernatants, any IL-6 activity described in this thesis is "IL-6-like" activity. Baseline spontaneous secretion of IL-6 by AM from saline aerosoled animals was approximately 10-15 units/ml. Spontaneous production was enhanced moderately but significantly by aerosol antigenic challenge at all times examined ($P < 0.03$). LPS challenge was associated with AM that secreted high levels of IL-6. Zymosan challenge led to significant increases in IL-6 levels in all groups ($P < 0.0001$); unstimulated or stimulated IL-6 production was highest in LPS-exposed animals and was higher in OA-challenged guinea pigs than in controls at all times examined ($P < 0.05$).

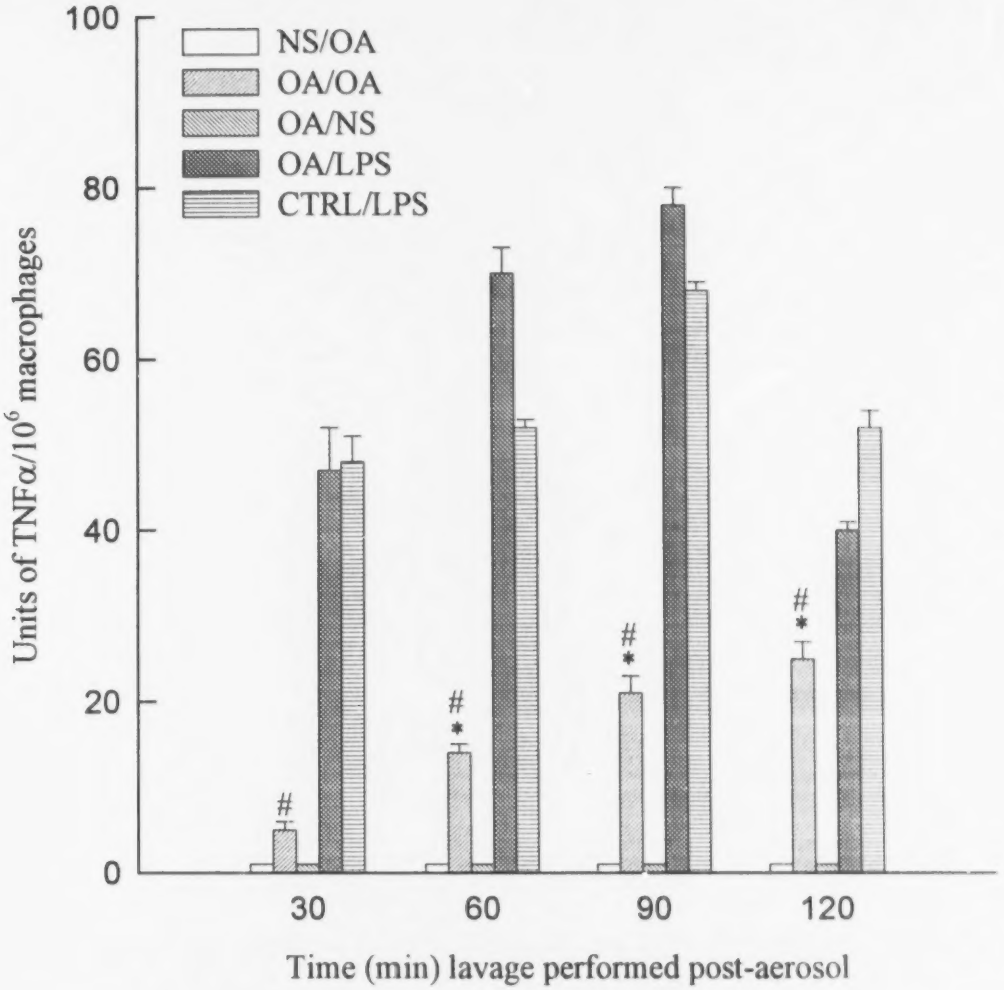

Figure 3.1. Spontaneous release of TNFα by guinea-pig AM. Guinea-pigs were divided into five groups: (A) (n = 8, OA/OA) received immune serum and inhaled OA aerosol; (B) (n = 8, OA/NS) received immune serum and inhaled saline aerosol; (C) (n = 4, NS/OA) received saline and inhaled OA aerosol; (D) (n = 4, OA/LPS) received immune serum and inhaled LPS; (E) (n = 4, CTRL/LPS) received control serum and inhaled LPS. 30, 60, 90, and 120 min post-aerosol, BAL was performed. AM were isolated by adherence and incubated for 18 h. Supernatants were collected and assayed for TNFα. * Denotes significantly different from time 30 min ($p < 0.05$, ANOVA). # Denotes significantly higher release in OA/OA group compared to NS/OA or OA/NS groups ($p < 0.05$, ANOVA).

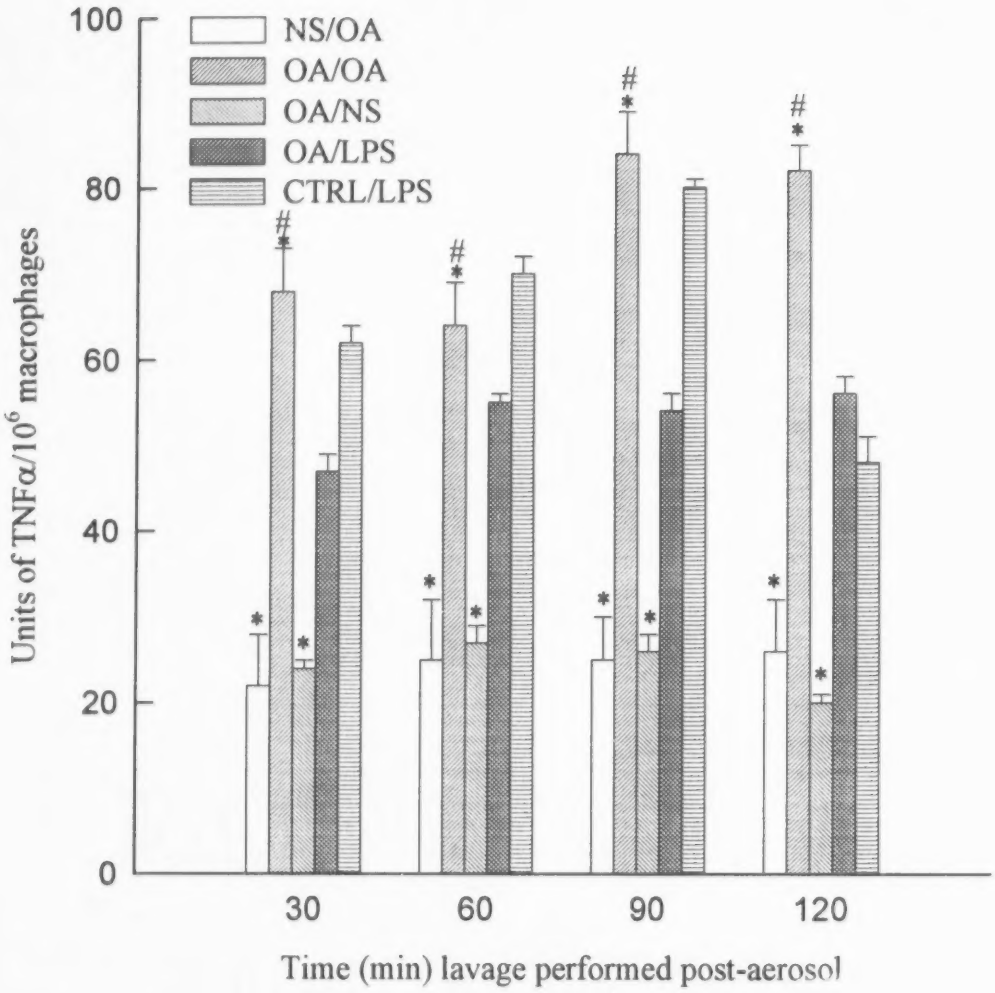

Figure 3.2. Zymosan-stimulated release of TNFα by guinea-pig AM. Experimental groups as indicated in the legend of Figure 3.1. * Denotes significantly different from unstimulated production ($p < 0.05$, ANOVA). # Denotes a higher release in OA/OA group compared to NS/OA or OA/NS groups ($p < 0.05$, ANOVA).

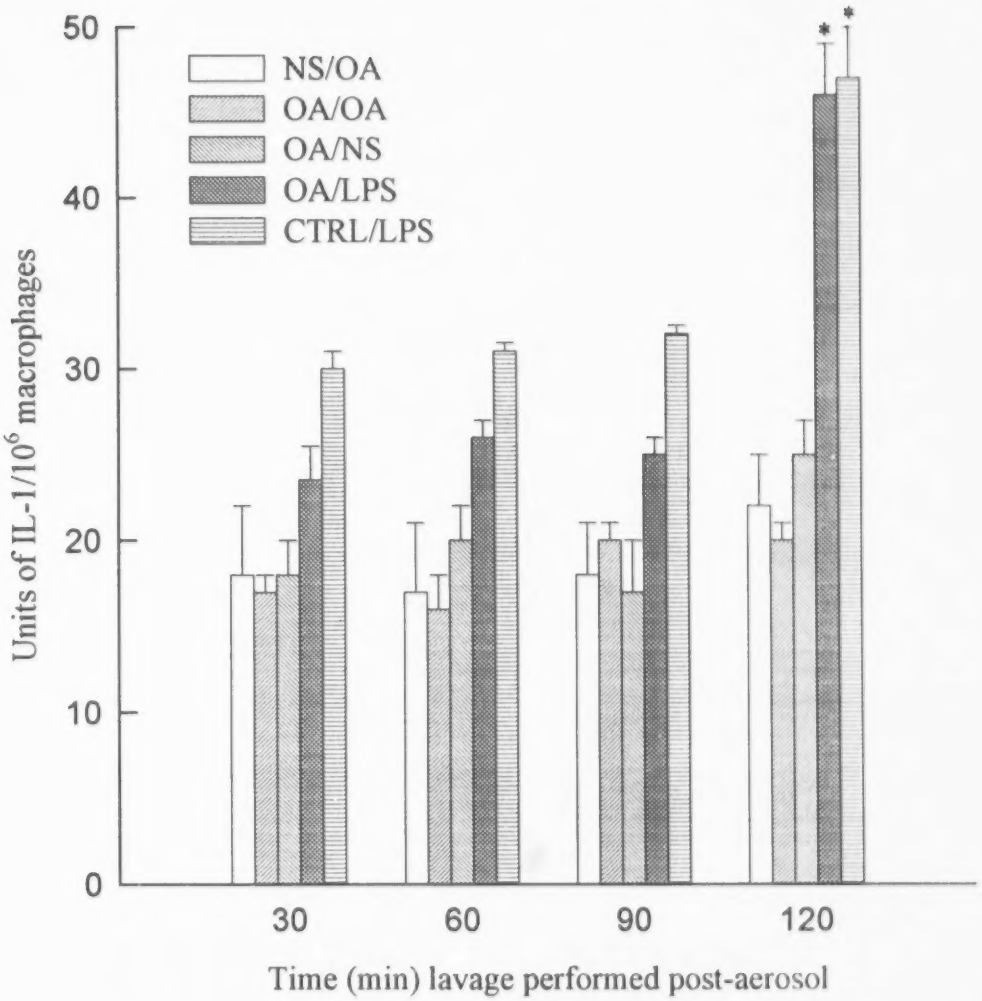

Figure 3.3. Spontaneous release of IL-1 by guinea-pig AM. Experimental groups as indicated in the legend of Figure 3.1. * Denotes greater release of IL-1 in these LPS-exposed groups, than all other groups at all times examined.

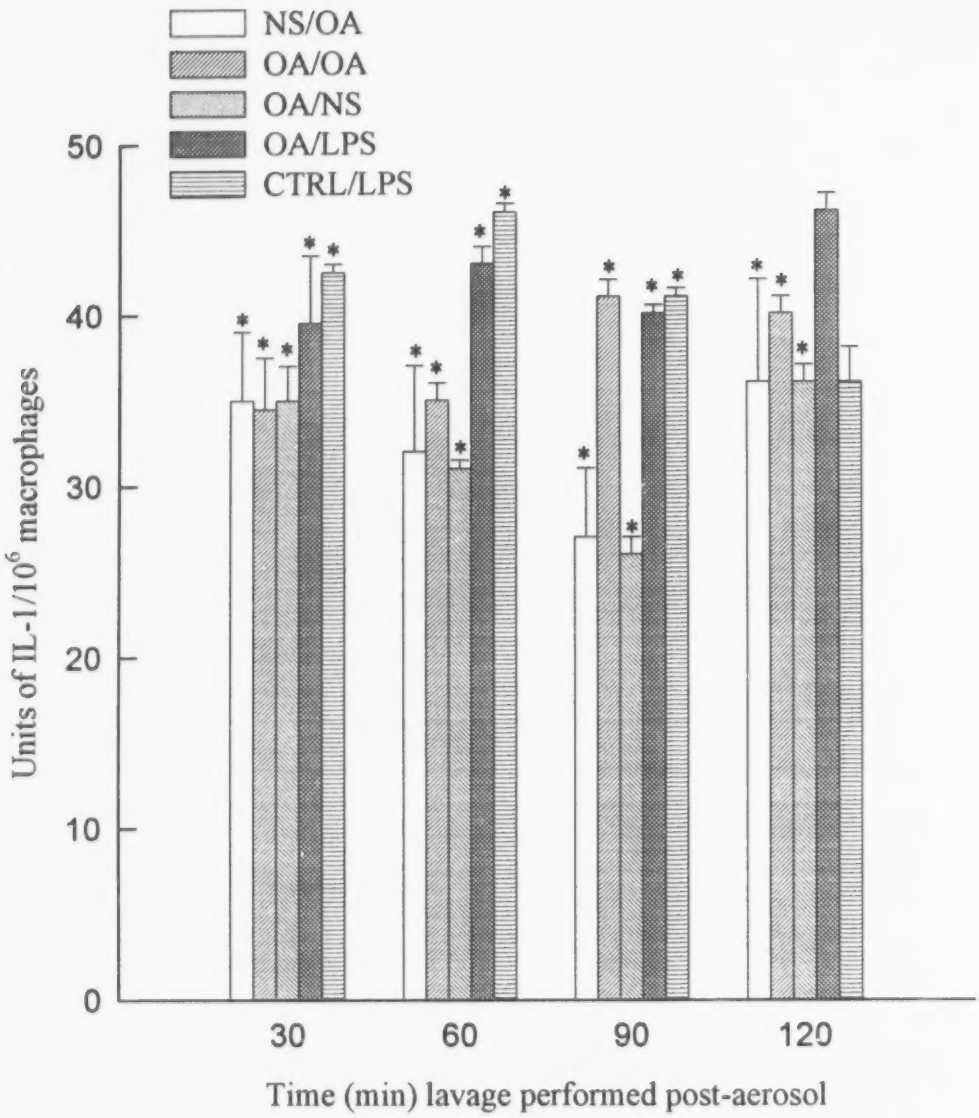

Figure 3.4. Zymosan-stimulated release of IL-1 by guinea-pig AM. Experimental groups as indicated in the legend of Figure 3.1. * Denotes significantly different from unstimulated production (see Figure 3.3) ($p < 0.05$, ANOVA).

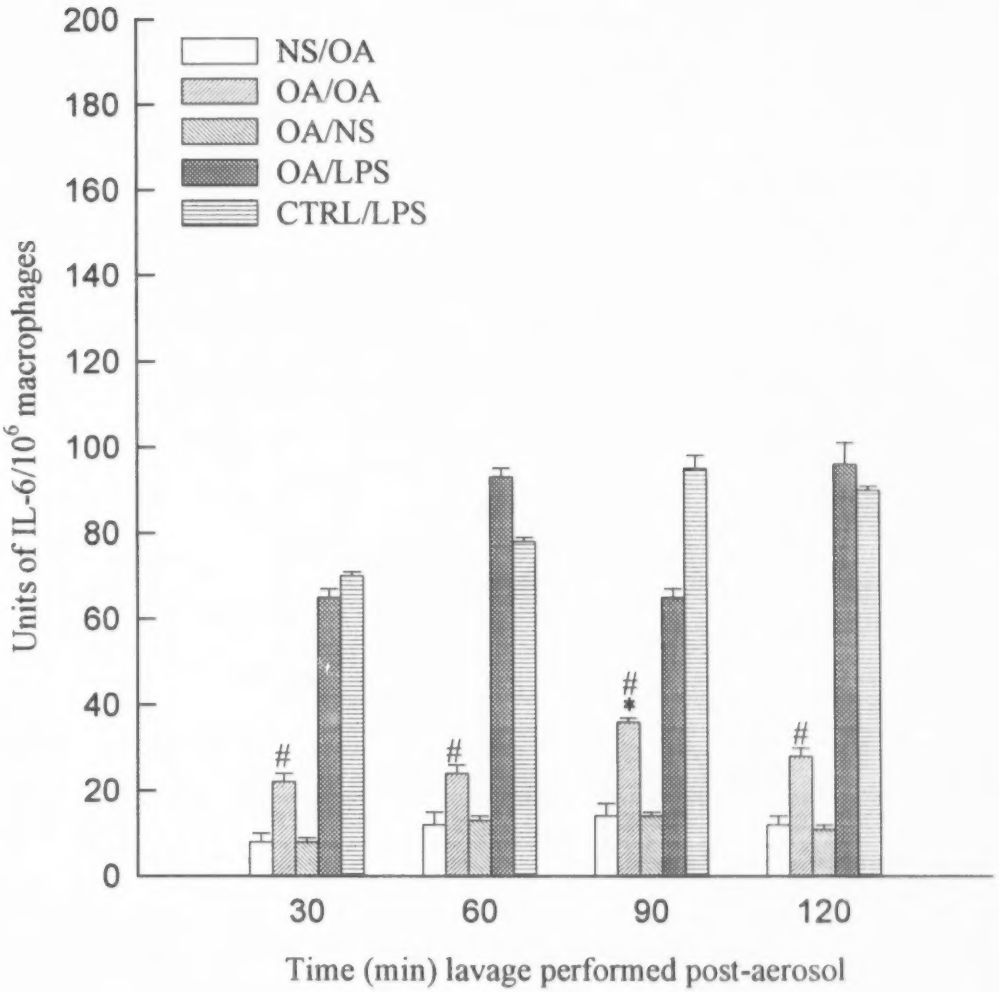

Figure 3.5. Spontaneous release of IL-6 by guinea-pig AM. Experimental groups as indicated in the legend of Figure 3.1. * Denotes significantly different from time 30 min ($p < 0.05$, ANOVA). # Denotes significantly higher release in OA/OA group compared to NS/OA or OA/NS groups ($p < 0.05$, ANOVA).

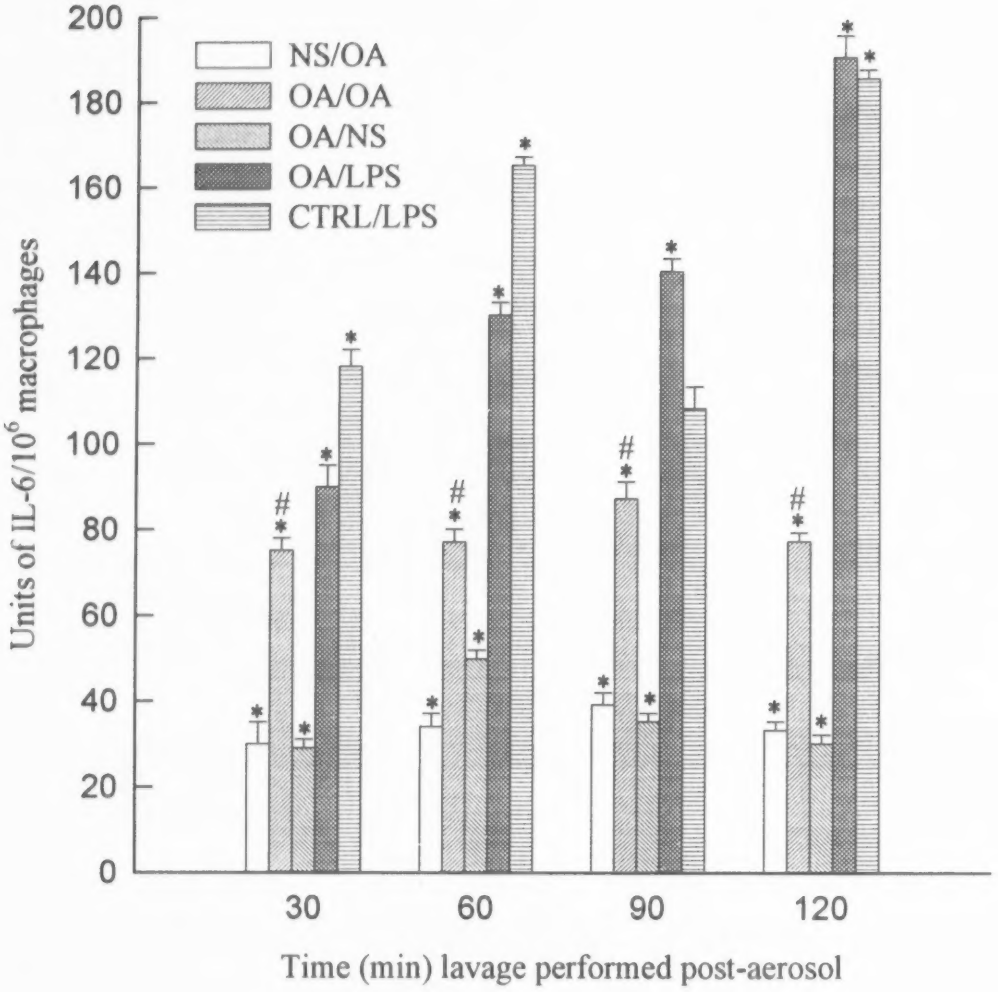

Figure 3.6. Zymosan-stimulated release of IL-6 by guinea-pig AM. Experimental groups as indicated in the legend of Figure 3.1. * Denotes significantly different from unstimulated production (see Figure 3.5) ($p < 0.05$, ANOVA). # Denotes a higher release in OA/OA group compared to NS/OA or OA/NS groups ($p < 0.05$, ANOVA).

DISCUSSION

This study examined monokine release in the early phase of an antigenic challenge in a guinea pig model of anaphylaxis. This challenge protocol has been shown to result in an increase in airway resistance (Ishida *et al.*, 1990), cellular influx (Pretolani *et al.*, 1988), and airway and tissue eosinophilia (Cerasoli *et al.*, 1991). Recently, this model has been used to study the role of neurokinins in mediating airway hyperresponsiveness (Ladenius & Biggs, 1991).

Cellular profiles in our different experimental groups were identical, with a majority of the free lung cells being macrophages, and a few being neutrophils, eosinophils and epithelial cells. Some authors have reported the presence of a significant number of eosinophils in the BAL cells of naive guinea-pigs (Dunn *et al.*, 1988). However, there are wide variations in the literature; they may reflect exposure to viral agents, housing conditions, etc. (Dunn *et al.*, 1988). We examined AM in the early phases of antigenic challenge, before dramatic changes in the cellular profile occur. The eosinophilic infiltrate that occurs in an antigenic challenge is evident 6-8 h after aerosol challenge (Dunn *et al.*, 1988), and the strong neutrophil influx follows the LPS aerosol challenge after 3-4 h (Venaille *et al.*, 1989).

The contribution of macrophages and their products in the early phase of a bronchial provocation model has not been the subject of close scrutiny. The inflammatory processes elicited by lung antigenic challenge are likely to be multifaceted and complex. The synthesis of mast cell mediators such as leukotriene C_4 (LTC_4) and platelet-activating factor (PAF) has been shown to initiate bronchoconstriction (Schleimer *et al.*, 1985). Our understanding of the role of mast cells is also expanding as these cells release cytokines, notably interleukin-4 (IL-4) and TNF (Plant *et al.*, 1989). *In vivo*, the predominant cell type in the airways and in the lung parenchyma is the macrophage.

This cell responds to a wide variety of signals and secretes numerous monokines that modulate the function of other leukocytes and structural cells. Recent data indicate that AM are readily activated to release oxidants, TNF, and IL-1 upon anti-IgE triggering (Joseph *et al.*, 1983; Rankin *et al.*, 1984; Gosset *et al.*, 1992b). Furthermore, bronchoalveolar macrophages obtained from asthmatic patients undergoing a late asthmatic reaction were shown to secrete high levels of IL-6 and TNF (Gossett *et al.*, 1991a).

Our study shows that an antigenic challenge resulted in AM that secrete increased amounts of IL-6 and TNFα (spontaneously and in response to zymosan) compared to macrophages from control animals aerosoled with saline, at all times examined. These high levels of IL-6 and TNFα were detected from AM that were collected from animals lavaged as early as 30 min post challenge. The spontaneous release of IL-1 remained constant, but zymosan stimulated greater release of IL-1 from antigen-exposed AM at 90 min post aerosol, compared to unchallenged groups in the same time periods. LPS challenge also resulted in secretion of large amounts of IL-6 and TNFα, both spontaneously and in response to stimulation by zymosan. Spontaneous IL-1 release by AM from LPS-challenged animals was enhanced only at 120 min post challenge. It remains to be seen whether this IL-1 production is related to the differences in the inflammatory cell influx between LPS-challenged (neutrophils) and antigen-challenged (eosinophils) guinea pigs. The roles of IL-1 and TNFα in neutrophil migration to an inflammatory site have been well described, *in vitro* and *in vivo* (Le & Vilcer, 1987). TNFα and IL-1 induce the expression and release of interleukin-8 (IL-8), which is a strong chemotactic factor for neutrophils (Baggiolini *et al.*, 1990). In addition, TNFα and IL-1 induce the expression of leukocyte adhesion molecules, which are essential for neutrophil migration (Gamble *et al.*, 1985). Our model is associated with a strong eosinophilia; the exact cytokine profile required for such an infiltrate is unclear, although

the contribution of PAF, LTC_4, and interleukin-5 (IL-5) are being examined (Sanderson, 1991).

Macrophage IL-6 and TNFα are thus released very early in a model of antigenic challenge. What stimulus could be responsible for eliciting this cytokine release? A direct nonspecific effect due to the nebulizing procedure can be excluded since animals nebulized with saline or albumin without prior sensitization failed to release TNFα spontaneously and released IL-6 in levels comparable to those of controls (i.e. non-treated animals, Group F, data not shown). IL-6 and TNFα are secreted by macrophages in response to a wide variety of agents, ranging from bacterial lipopolysaccharides to mineral dusts and immune complexes (Le & Vilcer, 1987). Expression of these monokines is also tightly controlled, and negative factors have been identified, such as steroids and IL-4 (Tevelde *et al.*, 1988). Given the very short time period that was required after exposure for enhanced TNFα and IL-6 releasability, the most likely candidates responsible for this increased release include IgE and/or IgG immune complexes (Rankin *et al.*, 1984) and the above-mentioned mast cell products. Involvement of mast cell products could be verified by treating guinea pigs with agents that stabilize mast cells, such as sodium cromoglycate (Pepys *et al.*, 1974). PAF, and possibly leukotrienes, have been shown to elicit TNF release from AM (Dubois *et al.*, 1989). Anti-IgE treatment of lung tissue slices results in the production of significant levels of TNF (Ohno *et al.*, 1990).

It remains a matter of speculation as to how production of these monokines may influence the inflammatory response elicited after antigen challenge. The proinflammatory properties of IL-1 and TNF are well described and are likely to contribute to the tissue-damaging reactions seen in this model. IL-1 increases histamine release from mast cells (Massey *et al.*, 1989) and elicits a large influx of neutrophils upon intratracheal

challenge (Ulich *et al.*, 1991). TNF is also involved in promoting inflammation by its ability to attract and activate neutrophils (Gamble *et al.*, 1985), enhance reactive oxygen intermediate release from macrophages, and stimulate fibroblast proliferation (Beutler & Cerami, 1987). Our data on monokine release after LPS inhalation are similar to those reported recently (Hogan & Vogel, 1988), with high levels of spontaneous TNF detectable early after inhalation and a subsequent enhanced IL-1 secretion. Moreover, in that study, no additional IL-1 activity was detected after *in vitro* LPS stimulation of AM from *in vivo* exposed guinea pigs (Hogan & Vogel, 1988). Our own findings show that cells from guinea pigs given LPS *in vivo* do not show dramatically enhanced IL-1 or TNFα synthesis after zymosan triggering; this may suggest an exhaustion of monokine precursors or the release of inhibitors of cytokines (Sekinger *et al.*, 1988). Cells from guinea-pigs challenged with antigen secreted higher levels of IL-1, IL-6, and TNFα upon zymosan stimulation, as compared to spontaneous production.

It is also worth mentioning that pretreatment of animals with the specific H_1 histamine receptor antagonist (pyrilamine maleate) 30 min prior to antigen challenge may have some influence on the AM subsequently collected. Both H_1 and H_2 receptors for histamine have been described on macrophages, while H_2 receptors predominate (Harris & Hutchison, 1994). Present literature does not describe the effects of pyrilamine on AM cytokine production. However, in AM collected from normal subjects, histamine has been shown to induce a dose- and time-dependent increase in cellular adhesion molecule expression (LFA-1, ICAM-1 and CD23b) and fibronectin release (*in vitro*), that is specifically blocked by the H_1 receptor antagonist, pyrilamine (Vignola *et al.*, 1994). Thus, it is possible that the administration of pyrilamine (ip) prior to antigen challenge and AM collection, may alter the phenotype of the AM subsequently retrieved.

In addition to its role as an inflammatory agent, TNF may directly mediate some

of the physiological consequences of antigenic challenge. Indeed, LPS-induced airway hyperresponsiveness in a rat model was found to be partly dependent on TNFα release (Kips *et al.*, 1992). The role of IL-6 in lung inflammation is less clear, with some data in experimental animals suggesting an anti-inflammatory role (Ulich *et al.*, 1991), perhaps based on the ability of IL-6 to downregulate TNF and IL-1 (Aderka *et al.*, 1989). The exact contribution of these monokines in the pathogenesis of this guinea pig model would best be delineated via the use of *in vivo* neutralizing antibodies. A recent publication has shown that, in a pulmonary anaphylaxis model, neutralization of endogenous levels of IL-1 inhibited the leukocyte influx and the antigen-induced airway hyperreactivity (Selig & Tocker, 1992).

In conclusion, our data suggest that an increase in resident AM production of IL-6 and TNF occurs after antigenic challenge in guinea pigs. This implicates these monokines in mediating some of the pathological aspects in this model of bronchial asthma.

CHAPTER 4

THE EFFECT OF CAPSAICIN PRETREATMENT ON TUMOR NECROSIS FACTOR α RELEASE FOLLOWING ANTIGEN CHALLENGE IN PASSIVELY IMMUNIZED GUINEA PIGS

SUMMARY

Experiments in Chapters 2 and 3 demonstrated the activation of AM to produce TNFα and IL-6 as a result of antigen challenge. This study tests the hypothesis that tachykinin neuropeptides may partly regulate TNFα production in our *in vivo* guinea-pig model of anaphylaxis. Guinea-pigs were pretreated with vehicle or capsaicin (a c-fiber neurotoxin that effectively eliminates neuropeptides (SP, NKA, NKB and CGRP) from c-fiber endings). Animals were then passively immunized with anti-OA antiserum and 24 h later inhaled aerosols of OA or LPS (as described in previous chapters). BAL was performed at 60, 90, or 120 min post aerosol, and AM were collected and incubated for 18 h, with or without zymosan. BAL fluid and cell supernatants were assayed for TNFα. TNFα was detected in the BAL fluid and in AM supernatants collected from animals that inhaled LPS as a positive control. TNFα levels in the BAL fluid from any of the pretreatment and then antigen challenged groups did not differ significantly. However, spontaneous and zymosan-stimulated production of TNFα from AM was significantly less in the immunized and then challenged group that had been pretreated with capsaicin when compared to the non-capsaicinized antigen challenged group. These data suggest that neuropeptides may play a role in regulating TNFα production from antigen activated guinea-pig AM. The effect of neuropeptides on TNFα secretion may be time-dependent as differences in TNFα production were not detected in the BAL fluid, but were apparent in AM supernatants collected after an 18 h incubation.

INTRODUCTION

The cytokines TNFα and IL-1 are secreted by activated macrophages, monocytes and mast cells (Sibelle & Reynolds, 1990; Gordon & Galli, 1990) and both have been proposed as potential contributors to the inflammatory changes seen in asthma. In support of this hypothesis, inhalation of TNFα aerosols increased bronchial reactivity to

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A version of this chapter has been published. Kelly DE, Denis M, Biggs DF. 1995. *Cell. Pharmacol.* 2, 423-428.

agonists in rats (Kips *et al.*, 1992). This effect was reduced significantly when anti-TNFα antibodies were administered 30 min before inhalation of TNFα. Also, soluble human IL-1 receptor has been shown to reduce the airways hyperresponsiveness that appears in actively immunized guinea-pigs challenged with large doses of antigen (Selig & Tocker, 1992). TNFα appears in bronchial alveolar lavage fluid (BALF) collected from guinea-pigs that have been passively immunized and challenged with antigen, during the early phase of the anaphylactic reaction (Kelly *et al.*, 1992). *In vitro*, human peripheral blood monocytes synthesize and secrete TNFα upon incubation with substance P (SP) or neurokinin A (NKA) (Lotz *et al.*, 1988). Also, SP, NKA and neurokinin B (NKB) induce superoxide formation by guinea-pig alveolar macrophages (AM) via actions involving NK-receptors (Brunelleschi *et al.*, 1990). In neuroglial cells, SP provides the second signal for TNFα secretion above the level induced by LPS alone. Anti-IL-1 antisera prevent or reduce this effect (Luber-Narod *et al.*, 1994). Treatment with the c-fiber selective neurotoxin capsaicin effectively eliminates neuropeptides from pulmonary c-fiber endings (O'Neill, 1991) and reduces the airways hyperresponsiveness seen in guinea-pigs that have been immunized against ovalbumin (OA) (actively or passively) and challenged by inhalation of OA aerosols (Ladenius & Biggs, 1989). Thus, there is good evidence suggesting that neuropeptide-induced modulation of TNFα and IL-1 secretion may occur in the early stages of the pathogenesis of asthma. As capsaicin pretreatment effectively prevents the develcpment of airways hyper-responsiveness in immunized and challenged animals, we determined whether the release of TNFα into BALF was altered in these animals compared with control groups. Also, we harvested AM from BALF of capsaicin pretreated and control groups of animals and compared their spontaneous and zymosan-induced release of TNFα. Inhalation of LPS aerosols was used a positive control in these experiments.

MATERIALS AND METHODS

Animals

As described in Chapter 2, page 45.

Capsaicinization

Methods for capsaicinization were followed as described in the literature (Biggs & Ladenius, 1990). On Day 1, the test animals were treated with pelargonic acid vanyllamide (synthetic capsaicin) in the following manner: First, they were lightly anesthetized with pentobarbital sodium (25-30 mg/kg, ip) and then injected with salbutamol (0.6 mg/kg, sc) to ensure some degree of bronchial dilation. Ten minutes later, they received capsaicin (10 mg/kg, sc, 12.5% solution in equal parts of Tween 80 and 95% ethanol, diluted to 25 mg/ml with normal saline). One hour later, they again received salbutamol (0.6 mg/kg, sc), followed by capsaicin (10 mg/kg, sc). This was repeated one hour later with the capsaicin dose increased to 30 mg/kg. A second dose of pentobarbital is usually required (10-20 mg/kg, ip) to maintain adequate anesthesia throughout the capsaicin procedure, however this was not always necessary. Epinephrine (0.1 ml, sc) was administered to those animals exhibiting marked respiratory distress. The control animals were treated similarly, but injected with the vehicle used for the capsaicin solution, or saline. After recovery from anesthetic, the animals were returned to their housing under laminar flow hoods.

Passive Immunization and Antigen Challenge

Seven days after capsaicinization, guinea pigs were passively sensitized to OA with guinea-pig hyperimmune serum (anti-OA antibody titre diluted to 1:2,000 in PBS (as shown by ELISA), 0.2 ml, ip), or normal saline (NS, 0.2 ml, ip). Twenty-four

hours later, all animals received mepyramine (0.5 mg/kg, ip) and 30 min later inhaled aerosols (Vix Acorn nebulizer, compressed air at 15 psi) of either: **A:** OA (2% in normal saline, up to 8 min). **B:** 0.9% saline, (8 min). **C:** LPS (*E. coli* 0111:B4. 150 ng/kg (in PBS) over 8 or 40 min)). 60, 90 or 120 min after challenge with OA, NS or LPS. The animals were anesthetized (pentobarbital, 40-50 mg/kg, ip), their tracheas cannulated (PE240) and BAL performed.

BAL Fluid and Cell Supernatant Collection and Storage

The lungs were lavaged with 2 x 5 ml PBS containing BSA (1%, to maintain viability of the cells recovered) and aprotinin (1000 KIU/ml, to inhibit protease activity). BAL fluids recovered were centrifuged (2,000 rpm, 5 min) and the supernatant decanted and immediately frozen at -20°C until assay. The cell pellet was resuspended in RPMI 1640 medium containing a penicillin (10,000 U/ml) and streptomycin (10 mg/ml) solution (1%), and stored at 4°C for < 48 h. Cells were plated at 1.5 x 10^6 cells/ml (1 ml) well in plastic 24-well culture plates. Cells were incubated 2 h at 37°C, 5% CO_2. Non-adherent cells were removed by vigorous washings with warm medium and adhered cells were more than 97% macrophages after this step as determined by Giemsa staining. Cells were then left untreated or stimulated with zymosan (1 mg/ml, Sigma), and incubated (18 h, 37°C, 5% CO_2) after which supernatants were filtered and frozen at -80°C until monokine assays were performed. To standardize cytokine production, adherent macrophages were dislodged from the plastic with cold EDTA solution and counted to allow expression of cytokines in U/10^6 AM. There were no significant differences in adherent macrophage numbers among the various experimental groups. Endotoxin contamination in the media was < 50 pg/ml, as indicated by the Limulus amoebocyte test (E-Toxate test, Sigma). All glassware was washed in E Toxa-Clean (Sigma) and baked overnight to destroy endotoxin.

TNFα Measurement

As described in Chapter 2, page 47.

Preparation of Guinea-Pig Sera

As described in Chapter 2, page 48.

Reagents and Chemicals

Actinomycin D, aprotinin, 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolinium bromide (MTT), chicken egg albumin (ovalbumin, Grade V) and LPS (*E. coli* 0111:B4) (Sigma, St. Louis, MO), penicillin-streptomycin mixture and RPMI 1640 medium (Gibco, Grand Island, NY), FBS (PA Biologicals, Sydney, Australia), recombinant human TNFα (Amersham Canada Ltd., Oakville, Ont.), rabbit anti-human TNFα and pre-immune serum (Olympus Corp., New York, NY).

Statistical Analyses

Data are expressed as mean ± SEM. Differences among the groups were examined using ANOVA, and assumed significant at the 5% level.

RESULTS

Effects of Capsaicin Pretreatment on Secretion of TNFα into BALF in Passively Immunized and Challenged Guinea-Pigs

Data are summarized in Table 4.1. In animals pretreated with capsaicin (50 mg/kg, sc) 7 d before experiments, secretion of TNFα into BALF was noted only in guinea-pigs that had been passively immunized with anti-ovalbumin antibody (α-OA-Ab)-containing serum (ip) and challenged 24 h later by inhalation of ovalbumin (OA,

2%) aerosols. Vehicle pretreated and saline pretreated control guinea-pigs passively immunized with α-OA-Ab-containing serum and challenged with OA aerosols also secreted TNFα into BALF. At 90 min after inhalation of OA aerosols, the amounts of TNFα secreted by these capsaicin, vehicle and saline treated groups were not statistically significantly different ($p > 0.05$). Capsaicin, vehicle and saline pretreated animals passively immunized with α-OA-Ab-containing serum that inhaled saline aerosols did not secrete detectable amounts of TNFα into BALF. Also, capsaicin pretreated guinea-pigs passively immunized with saline that inhaled OA aerosols did not secrete TNFα into BALF.

Effects of Capsaicin Pretreatment on Secretion of TNFα into BALF in Guinea-Pigs that Inhaled LPS

Data are summarized in Table 4.2. Animals pretreated with capsaicin, vehicle, or saline and passively immunized with α-OA-Ab-containing serum that inhaled LPS aerosols for 40 min secreted similar amounts of TNFα into BALF at 90 min post-aerosol inhalation. LPS inhalation for 8 min induced secretion of TNFα in capsaicin pretreated animals passively immunized with saline. The amounts of TNFα secreted by these capsaicin, vehicle and saline treated groups that had inhaled LPS for 40 or 8 min were not statistically significantly different ($p > 0.05$).

Effects Of Capsaicin Pretreatment On Spontaneous Secretion Of TNFα By AM Recovered From BALF from Passively Immunized and Challenged Guinea-Pigs

Data are summarized in Table 4.3. AM were isolated from BALF by adherence and incubated for 18 h. Amounts of TNFα spontaneously secreted by AM recovered from BALF of the capsaicin pretreated guinea-pigs passively immunized with α-OA-Ab-

containing serum and challenged with OA aerosols were significantly less than those from vehicle and saline pretreated animals ($p < 0.05$). AM recovered from BALF of similarly treated control animals that had inhaled saline aerosols spontaneously released amounts of TNFα that were barely detectable or below the detection limit (2 U/ml) of the assay for TNFα.

Effects of Capsaicin Pretreatment on Spontaneous Secretion of TNFα by AM Recovered from BALF from Guinea-Pigs that Inhaled LPS

Data are summarized in Table 4.4. Amounts of TNFα spontaneously secreted by AM recovered from BALF of the capsaicin, vehicle and saline pretreated guinea-pigs passively immunized with α-OA-Ab-containing serum or saline and exposed to LPS aerosols for 40 or 8 min were similar and not significantly different ($p > 0.05$).

Effects of Capsaicin Pretreatment on Zymosan-Induced Secretion of TNFα by AM Recovered from BALF from Passively Immunized and Challenged Guinea-Pigs

Data are summarized in Table 4.3. Amounts of zymosan-induced-TNFα secreted by AM recovered from BALF of capsaicin pretreated guinea-pigs passively immunized with α-OA-Ab-containing serum and challenged with OA aerosols were significantly less than those from AM obtained from vehicle and saline pretreated animals that had also been challenged with antigen ($p < 0.05$). Zymosan stimulation did not increase significantly the amount of TNFα secreted spontaneously by AM in any of these three groups. By contrast, zymosan stimulated AM recovered from BALF of similarly treated animals that had inhaled saline aerosols released amounts of TNFα that were significantly greater than the minimal amounts these AM released spontaneously. These amounts were similar to those released by AM from capsaicin pretreated, passively

immunized and challenged animals.

Effects of Capsaicin Pretreatment on Zymosan-Induced Secretion of TNFα by AM Recovered from BALF from Guinea-Pigs that Inhaled LPS

Data are summarized in Table 4.4. Zymosan stimulation did not significantly alter the amounts of TNFα secreted spontaneously by AM recovered from BALF of the capsaicin, vehicle and saline pretreated guinea-pigs passively immunized with α-OA-Ab-containing serum or saline and exposed to LPS aerosols for 40 or 8 min.

TABLE 4.1

Effects of capsaicin pretreatment on secretion of TNFα into BALF from passively immunized and challenged guinea-pigs. Groups of animals were pretreated with capsaicin, vehicle or saline and then immunized with α-OA-Ab or saline. 24 h later they inhaled aerosols of OA or saline (over 8 min) and 90 min later their lungs were lavaged. Concentrations of TNFα detected in the BALF (using the L929 cytotoxic assay) are expressed as the average U/ml ± SEM.

Group #	t (min)	n	Pretreatment	Immunization	Aerosol	TNFα, U/ml
1	90	5	Capsaicin	α-OA-Ab	OA	41 ± 12
2	90	3	Vehicle	α-OA-Ab	OA	32 ± 7
3	90	4	Saline	α-OA-Ab	OA	29 ± 3
4	90	4	Capsaicin	α-OA-Ab	Saline	ND
5	90	4	Vehicle	α-OA-Ab	Saline	ND
6	90	4	Saline	α-OA-Ab	Saline	ND
7	90	4	Capsaicin	Saline	OA	ND
8	90	4	Capsaicin	Saline	OA	ND

ND = none detectable.

TABLE 4.2

Effects of capsaicin pretreatment on secretion of TNFα into BALF from guinea-pigs that inhaled LPS. Groups of animals were pretreated with capsaicin, vehicle or saline and then immunized with α-OA-Ab or saline. 24 h later they inhaled aerosols of LPS (over 8 or 40 min). 60, 90 or 120 min later their lungs were lavaged. Concentrations of TNFα detected in the BALF (using the L929 cytotoxic assay) are expressed as the average U/ml ± SEM.

Group #	t (min)	n	Pretreatment	Immunization	Aerosol	TNFα, U/ml
9	90	2	Capsaicin	α-OA-Ab	LPS	24 ± 9
10	90	2	Vehicle	α-OA-Ab	LPS	17 ± 10
11	90	2	Saline	α-OA-Ab	LPS	22 ± 9
12	60	4	Capsaicin	Saline	LPS*	15 ± 5
13	120	4	Capsaicin	Saline	LPS*	21 ± 8

*LPS aerosol inhaled for 8 min.

TABLE 4.3

Effects of capsaicin pretreatment on spontaneous and zymosan stimulated secretion of TNFα by AM recovered from BALF from passively immunized and challenged guinea-pigs. Groups of animals were pretreated with capsaicin, vehicle or saline and then immunized with α-OA-Ab or saline. 24 h later they inhaled aerosols of OA or saline (over 8 min) and 90 min later their lungs were lavaged. AM were collected and incubated for 18 h, with or without zymosan. Supernatants were collected and assayed for TNFα using the L929 cytotoxic assay. TNFα concentrations are expressed as the average U/10^6 AM ± SEM.

Group #	t (min)	n	Pretreatment	Immunization	Aerosol	Spontaneous TNFα, U/10^6 AM	Zymosan-Induced TNFα, U/10^6 AM
1	90	5	Capsaicin	α-OA-Ab	OA	29 ± 13*	32 ± 13*
2	90	3	Vehicle	α-OA-Ab	OA	63 ± 12	60 ± 14
3	90	4	Saline	α-OA-Ab	OA	53 ± 7	61 ± 9
4	90	4	Capsaicin	α-OA-Ab	Saline	2 ± 1	28 ± 13
5	90	4	Vehicle	α-OA-Ab	Saline	ND	26 ± 15
6	90	4	Saline	α-OA-Ab	Saline	ND	17 ± 5
7	90	4	Capsaicin	Saline	OA	ND	18 ± 4
8	90	4	Capsaicin	Saline	OA	4 ± 1	22 ± 7

ND = none detectable.

* $p < 0.05$, Group 1 *vs* Groups 2 or 3.

TABLE 4.4

Effects of capsaicin pretreatment on spontaneous and zymosan stimulated secretion of TNFα by AM recovered from BALF from guinea-pigs that inhaled LPS. Groups of animals were pretreated with capsaicin, vehicle or saline and then immunized with α-OA-Ab or saline. 24 h later they inhaled aerosols of LPS (over 8 or 40 min). 60, 90 or 120 min later their lungs were lavaged. AM were collected and incubated for 18 h, with or without zymosan. Supernatants were collected and assayed for TNFα using the L929 cytotoxic assay. TNFα concentrations are expressed as the average U/10^6 AM ± SEM.

Group #	t (Min)	n	Pretreatment	Immunization	Aerosol	Spontaneous TNFα, U/10^6 AM	Zymosan-Induced TNFα, U/10^6 AM
9	90	2	Capsaicin	α-OA-Ab	LPS	46 ± 13	39 ± 21
10	90	2	Vehicle	α-OA-Ab	LPS	31 ± 17	58 ± 13
11	90	2	Saline	α-OA-Ab	LPS	52 ± 6	61 ± 19
12	60	4	Capsaicin	Saline	LPS*	18 ± 2	37 ± 10
13	120	4	Capsaicin	Saline	LPS*	26 ± 13	45 ± 19

*LPS aerosol inhaled for 8 min.

DISCUSSION

Cytokine-mediated interactions among macrophages, lymphocytes and eosinophils appear to be involved in the pathogenesis of the eosinophilia and airways' inflammation that characterize asthma. TNFα is chemotactic for monocytes and polymorphonuclear leukocytes, activates endothelial cells leading to margination, vascular leakage and eosinophilia, and stimulates B-lymphocyte transformation to plasma cells (Kroegel *et al.*, 1992; Brown *et al.*, 1990; Camussi *et al.*, 1991; Schollmeier, 1991; Semenzato, 1990). We have recently shown that TNFα was released from the lungs of passively sensitized guinea pigs as early as 60 min post-antigen inhalation (Kelly *et al.*, 1992), and suggested a role for this cytokine as one of the early mediators in the inflammatory processes associated with asthma. Neurogenic inflammation mediated by neuropeptides such SP, NKA, and calcitonin-gene-related-peptide (CGRP) has been demonstrated in rat and guinea-pig airways (Barnes, 1991). The peptides are released from sensory c-fibers upon stimulation by mediators such as histamine, leukotrienes and bradykinin, by mechanical irritation and by axonal reflexes, and mediate protein extravasation, vasodilation, mucous secretion and contraction of smooth muscle in the airways (Lundberg *et al.*, 1987; Lundberg & Saria, 1982; Lundbald *et al.*, 1983). Moreover, human blood monocytes (precursors to alveolar macrophages) synthesize and secrete IL-1 and TNFα when incubated *in vitro* with SP and NKA (Lotz *et al.*, 1988) and SP, NKA and NKB have been shown to activate guinea-pig AM to produce superoxide anion via NK-receptor interactions (Brunelleschi *et al.*, 1990).

The findings summarized in Table 4.1 confirm that challenge by inhalation of OA aerosols in guinea-pigs passively immunized against OA results in the secretion of TNFα into BALF. Amounts released were similar in capsaicin, vehicle and saline pre-treated animals. Thus, capsaicin pretreatment does not alter the amount of TNFα that appears in

BALF acutely after antigen challenge suggesting that neuropeptide depletion does not influence TNFα secretion in this respiratory model depicting early events of asthma. In control groups of guinea-pigs, pretreatment with capsaicin, vehicle or saline and passive immunization with α-OA-Ab, followed by challenge with saline aerosols did not induce any TNFα secretion. This shows clearly that challenge with specific antigen is essential for secretion of TNFα into BALF. This finding was confirmed by the lack of TNFα secretion into BALF from guinea-pigs that inhaled OA aerosols after pretreatment with capsaicin and passive immunization with saline. Also, this experiment shows that inhalation of OA aerosol itself does not induce TNFα secretion.

The data summarized in Table 4.2 were obtained primarily for control purposes. When LPS inhalation was used as a positive control, TNFα appeared in BALF in similar amounts whether the animals had been pretreated with capsaicin, vehicle or saline and had been passively immunized with serum containing α-OA Ab or saline. Thus, by contrast with experiments done *in vitro* with neuroglial cells (Luber-Narod *et al.*, 1994), our findings suggest that neuropeptides found in pulmonary c-fiber endings have no role in modulating antigen or LPS-induced TNFα secretion into BALF in this animal model.

The spontaneous and zymosan-induced release of TNFα was examined in studies of AM harvested from BALF. Data are summarized in Tables 4.3 and 4.4. AM from guinea-pigs pretreated with capsaicin and passively immunized against OA and challenged with OA aerosol released less TNFα spontaneously and in response to zymosan stimulation than AM from vehicle or saline treated controls. Thus, capsaicin pretreatment reduced the ability of AM to synthesize and release this cytokine. Interestingly, AM must be maximally stimulated for TNFα secretion by the passive immunization and challenge as zymosan did not increase secretion of this cytokine. In control groups pretreated similarly but which inhaled saline aerosols, there was no

spontaneous release of TNFα. However, zymosan stimulated these cells to secrete TNFα in generally lower amounts than those seen after antigen challenge. Zymosan failed to increase TNFα secretion by AM from any of the groups that had inhaled LPS. This suggests that these cells are maximally stimulated by LPS. In animals that had inhaled LPS, capsaicin pretreatment did not alter spontaneous or zymosan-stimulated TNFα release by AM.

Although we reported detection of TNFα in BALF from immunized challenged guinea-pigs between 30 and 60 min after challenge (Kelly *et al.*, 1992) and associated this with the early asthmatic reaction, Gosset *et al.* (1992) detected TNFα in BALF, from humans only during late reactions. Our solution used for lavage contained an enzyme inhibitor (aprotinin) in order to prevent breakdown of cytokines by proteases and TAME-esterases that are released from cells such as mast cells during the early asthmatic reaction. This therefore, may account for the difference between the observations.

Rabbit and human AM express IgE and IgG receptors (Kindt *et al.*, 1991; Speigelberg *et al.*, 1983) and IgE-and IgG-mediated reactions induce TNFα synthesis and secretion (Ohno *et al.*, 1990; Wershil *et al.*, 1991). The serum used in our experiments contained no IgE as determined by passive cutaneous anaphylaxis tests. ELISA indicated that the serum contained only α-OA-Ab of IgG isotypes (data not shown). Thus, it is likely that IgG/Fc receptor interaction mediated the TNFα secretion observed in these studies. Elimination of neuropeptides from pulmonary c-fiber nerve endings appears to have no effect upon the amounts of TNFα appearing in BALF after antigen challenge or LPS inhalation. However, it reduced both spontaneous and zymosan-induced release of this cytokine from incubated AM. It is possible that during the acute anaphylactic reaction TNFα is released by cells other than AM. Mast cells contain preformed TNFα (Gordon & Galli, 1990). However, they may not be present in

sufficient numbers to generate the amount of TNFα found in BALF. Also, mast cells degranulate within minutes and TNFα is first detectable in BALF at 30-60 min post-challenge. It seems that these cells are unlikely to be the source of the TNFα detected, as lavage was performed in this experiment at 90 min post-aerosol.

The airway hyperreactivity that follows immunization and challenge is diminished by capsaicin pretreatment (Ladenius & Biggs, 1989). Thus, this may relate to the observed decrease in production or secretion of TNFα (a pro-inflammatory cytokine) from AM collected and cultured from capsaicinized guinea pigs. This is in agreement with other findings (Kips *et al.*, 1992) that suggest the involvement of TNFα in the pathogenesis of airway disease. The concentrations of TNFα in the lavage fluids among the three pretreatment groups that had been challenged with antigen, were not significantly different from each other. This may reflect a time course for neuropeptide involvement in TNFα secretion. There is an initial production and release of TNFα into the lavage fluid 90 min post-antigen challenge, and neuropeptides released or formed may then offer a second signal to AM for the continued or later production of TNFα.

We conclude that in this passively sensitized and then antigen challenged guinea-pig model of asthma, spontaneous and zymosan-stimulated production of TNFα from cultured AM is suppressed by pretreating animals with the neurotoxin, capsaicin.

CHAPTER 5

TUMOR NECROSIS FACTOR α SECRETION BY GUINEA-PIG ALVEOLAR MACROPHAGES DURING ISOLATION BY ADHERENCE

SUMMARY

To further investigate the production of TNFα from guinea-pig AM, an "in-house" *in vitro* AM cell-culture model was established and refined, as was the L929-bioassay used for detection of TNFα. Eight sequential 2 x 5 ml (i.e. 10 ml) lavages were performed on naive guinea-pigs. Total and differential cell counts were obtained for each 10 ml lavage aliquot so that the cellular make-up of the BAL fluid was determined (see text for results). Next, because AM are routinely isolated from BAL fluid by their selective adherence to plastic, experiments were done to determine if this causes TNFα production. BAL was performed on groups of guinea-pigs with complete medium or with complete medium containing the endotoxin inactivator, polymyxin B (as a control for endotoxin). Cells within each groups were combined, plated and incubated under adherent or non-adherent conditions. Cell supernatants were collected at various times and assayed for TNFα. Findings indicate that upon adherence to plastic, AM secrete significantly higher levels of TNFα than non-adhered cells. Also, upon LPS stimulation, adhered cells produce more TNFα than non-adhered cells. We were unable to detect any preformed TNFα from freshly isolated then lysed AM. Thus, isolation of AM by adherence to plastic induces TNFα production and may lead to biochemical changes in the cell that must be considered in studies, *in vitro*.

INTRODUCTION

Pulmonary macrophages form one of the lungs' first lines of defence against insults. Macrophages secrete a variety of cytokines following exposure to stimuli such as endotoxins, phorbol myristicate, zymosan and immunoglobulins. Macrophages are found in large numbers within the distal airways and far outnumber other cell types such as neutrophils, eosinophils and lymphocytes (Sibelle & Reynolds, 1990). Cytokine-mediated interactions among macrophages, lymphocytes and eosinophils may be involved in the pathogenesis of the eosinophilia and airways' inflammation that characterizes asthma (Brown *et al.*, 1990; Camussi *et al.*, 1991; Schollmeier, 1991;

Semenzato, 1990).

Pulmonary alveolar macrophages are readily collected by bronchial alveolar lavage and are commonly isolated from the lavage fluid by adherence to plastic. However, this isolation procedure may induce cytokine formation. Within 30 min of human monocyte adherence to plastic, there were selective increases in mRNA levels for c-fos and the pro-inflammatory cytokine, tumor necrosis factor α (TNFα) (Haskill *et al.*, 1988). By contrast, adherence was not required for induction of high levels of interleukin 1 (IL-1) mRNA. Although cytokine mRNA induction occurred in this experiment, a second "activating" signal (lipopolysaccharide) was required for secretion of TNFα and IL-1. Others (Fuhlbrigge *et al.*, 1987) have shown that the stimulation of murine peritoneal exudate cells by adherence induced expression of intracellular, membrane and extracellular IL-1 activity within 4 h. Analysis of mRNA from these cells showed concurrent induction of both IL-1α and IL-1β mRNA within 1 h.

In this chapter, we compared the production of TNFα from guinea-pig AM that have been incubated while adhering to plastic, to AM that were incubated under non-adherent conditions. Also, we determined whether freshly isolated guinea-pig AM contained pre-formed TNFα.

MATERIALS AND METHODS

Animals

As described in Chapter 2, page 46.

Lavage

Guinea-pigs were anesthetized with pentobarbital (50 mg/kg, ip). When deeply anesthetized, a cannula (4 cm long, PE240) was inserted into the trachea about 1 cm

proximal to the larynx and an 18 G by 1.5 inch hypodermic needle inserted into the cannula and sealed in place via a short length of flexible tubing (Masterflex 96400-14). Using a 5-ml luer lock syringe, the lungs were lavaged with warm (37°C) RPMI 1640 medium (FBS, 10%; L-glutamine, 2 mM; penicillin/streptomycin solution 1%, without or with polymyxin B, 1 or 10 μg/ml) by infusing the medium over 2 s, waiting 2 s, then withdrawing the medium over 2-3 s until no more could be aspirated. This procedure was repeated for a total of 16 x 5-ml lavages. Fluid from each lavage were combined into 50 ml polypropylene (Falcon 2098 Blue Max) conical tubes and stored on ice until being centrifuged (2,000 rpm, 5 min, Dynac II, Adams). Cell pellets were resuspended in the same media, then counted (Neubauer hemocytometer, trypan blue exclusion stain) and adjusted to 10^6 cells/ml.

Cell Types

Sequential 10 ml lavages (2 x 5 ml) were performed on 2 guinea-pigs using 1% bovine serum albumin in phosphate buffered saline (pH 7.4). Total cell counts were made on each sequential 10 ml aliquot (Turks or trypan blue). Differential slides (using Cytospin and staining with Leukostat) were also made with each 10 ml aliquot and differential cell counts done on 400 cells from the aliquot of each animal. Data are expressed as the combined average ± SE for each 10 ml.

Storage of Pre-formed TNFα

Lavage was performed on two guinea-pigs using standard media. A portion of the cells recovered were combined, pelleted and resuspended at 10^6 cells/ml and immediately lysed by freezing at -20°C and then thawing. Other samples were plated at 10^6 cells /well (1 ml in each of 4 wells) in 24 well plates and allowed to adhere for 2 h, after which they were washed 3 times with media and 1 ml of standard media was added

prior to freezing. Another group of cells were allowed to adhere for 2 h, washed 3 times with warm media, new media added and the cells incubated for a further 2 h, followed by lysis into fresh media. Supernatants were centrifuged after thawing and samples were then assayed in duplicate for TNFα.

Isolation of Cells by Adhesion

Guinea-pigs were lavaged with standard medium (n = 2), or medium containing polymyxin B, 1.0 μg/ml (n = 2) or 10.0 μg/ml (n = 2). Cells from each group were combined and resuspended in the appropriate medium. Cells were plated into 24 well cell culture plates (1 ml/well, 4 wells for each sample) and incubated for 2 h (37°C, 5% CO_2). Supernatants were collected and the cells washed 3 times with warm medium. Medium was replaced (1 ml/well), collected with replacement every 30 min thereafter over the next 4 h, centrifuged, and then frozen until assayed for endotoxin and TNFα. Cells were stained with trypan blue at the end of the experiment to determine whether a possible decline in TNFα secretion was due to cell death.

Treatment of Non-adhered Cells

Guinea-pigs were lavaged as above. The majority of cell types other than macrophages were eliminated by discarding the first 10 ml of lavage fluid. The cell pellet was resuspended in the appropriate media, incubated in 50 ml polypropylene culture tubes (4 tubes in each group) and constantly rocked during incubation (using a plate shaker) to minimize adhesion and aggregation. Samples of media containing cells were withdrawn at 2 h post the start of incubation and at every 30 min over the following 4 h. All supernatants collected were centrifuged and frozen at -80°C until assay for endotoxin and TNFα.

Activation of Macrophages with LPS

Two guinea-pigs were lavaged with standard medium. Macrophages isolated by adherence were incubated with doses of LPS (*E. coli* 0111:B4, 0.01, 0.1, 1.0, or 10.0 μg/ml) in medium, 2 h following a 2 h adherence period, to assess the cells' ability to become activated post adherence. Macrophages incubated in polypropylene tubes were incubated with doses of LPS for a period of 2 h (after an initial 2 h incubation), after which supernatants were collected and assayed for TNFα.

TNFα Measurement

Levels of TNFα were detected using an adaptation of the L929 bioassay (Branch *et al.*, 1991). Briefly, L929.8 cells (in standard supplemented RPMI-1640 media) were plated into 96 well microtiter plates at 100,000 cells per well (75 μl) and the cells incubated and allowed to adhere overnight. Cells were then treated with Actinomycin D (8 μg/ml in complete standard media (25 μl)), for 2 h. After which, dilutions of sample and standard (r(h)TNFα (Genzyme)) were added to wells, in duplicate and incubated. 24 h later, 50 μl of neutral red dye (0.05% in normal saline) was added to each well and the plates incubated for 2 h. Plates were then carefully inverted, emptied and washed once with PBS (200 μl). The cell layer was lysed with 0.05 M NaH_2PO_4 (in 50% v/v ethanol, 100 μl) and mixed on a plate shaker for 20 min. The optical density of the wells was read on an ELISA reader (Softmax) at 540 nm. TNFα concentrations in the supernatants were determined from a standard curve estabiished on each plate with r(h)TNFα (Genzyme). The cytotoxic activity in the samples and standard curve was blocked using rabbit-anti-human-TNFα serum (50 μl) (Genzyme).

Statistical Analyses

Data were analyzed on an IBM computer using SigmaStat statistical program. Differences among treatment groups (i.e. standard medium and polymyxin B medium) were analyzed using ANOVA. The Students "t" test was used to analyze differences among cell type numbers, as well as TNFα concentrations in cell lysates and in supernatants after LPS activation. Data were considered statistically significant if p values were less than 0.05. Data are expressed as mean ± SE.

Materials

Polymyxin B and the Limulus Amebocyte Lysate kit (E-Toxate) were purchased from Sigma (Mississauga, Ontario). All media and supplements were purchased from GIBCO BRL (Burlington, Ontario) and the cytokine research products from Genzyme Diagnostics (Cambridge, MA, USA).

RESULTS

Cell Types

Counts of cells obtained from 5 ml lavages that had been combined into sequential 10 ml aliquots (total lavaging volume of 80 ml) showed that about $3.1 \pm 0.565 \times 10^7$ cells are found in the lavage fluid of each guinea-pig after staining with trypan blue and $2.2 \pm 0.256 \times 10^7$ cells when staining with Turk's stain (Figure 5.1). The greatest number of cells appeared within the first 50-60 ml of lavage fluid; cell numbers tapered off thereafter.

Differential cell counts on each 10 ml aliquot of lavage showed that the highest number of cells other than macrophages, was found in the first 10 ml of lavage fluid (Figure 5.2). About 16% of the cells in the first 10 ml of lavage fluid were eosinophils,

neutrophils and lymphocytes, compared to only about 7% in subsequent 10 ml lavage aliquots.

Storage of Pre-formed TNFα

TNFα was not detected in the BAL fluids or in the lysate from freshly isolated, then lysed AM. However, after incubation for 2 h under adherent or non-adherent conditions, TNFα concentrations in lysates were about 7.6 ± 2.9 pg/ml/10^6 non-adherent cells and 11.6 ± 4.4 pg/ml/10^6 adherent cells (Figure 5.3). After cells were incubated for 4 h, lysates from adhered then lysed cells contained significantly higher concentrations of TNFα compared to supernatants from non-adhered cells (56.2 ± 6.5 pg/ml/10^6 adherent cells versus 7.1 ± 2.0 pg/ml/10^6 non-adherent cells (* $p < 0.001$)). Also, significantly more TNFα was found in lysates from adhered cells after 4 h incubation than a 2 h incubation (# $p < 0.001$). Overall however, the amounts of TNFα detected in this experiment are low, compared to those induced by adherence or particularly, LPS. In the L929 cell-based assay used to detect TNFα, the cytotoxic activity in these and subsequent samples was prevented using rabbit-anti-human-TNFα serum (50µl) (Genzyme), confirming TNFα bioactivity in these samples.

TNFα Production from AM Isolated by Adhesion

TNFα was not detected in BAL fluid at the time of lavage (time 0). However, after adherence for 2 h, levels of TNFα in supernatants averaged 279.3 ± 89.3, 235.2 ± 37.1 and 295.2 ± 47.9 pg/ml/10^6 cells, in the standard medium group and the polymyxin B (1 or 10 µg/ml) groups, respectively (Figure 5.4). Amounts of TNFα in supernatants gradually declined with time and were low in all groups after adherence for 4 h. Trypan blue staining for non-viable cells showed that cells remained viable throughout the experiment, thus the decline in TNFα levels is not due to cell death.

TNFα Production from Non-adhered Cells

TNFα was not detected in the lavage fluid at the time of lavage (time 0). In all groups and time periods examined thereafter, TNFα levels in supernatants were negligible (ranging from non-detectable to 18 pg/ml/10^6 non-adhered cells). TNFα levels in non-adhered AM supernatants were significantly less than in adhered AM supernatants at time 2 h, through to 6 h ($p < 0.001$) (Figures 5.4 and 5.5). Trypan blue staining for non-viable cells showed that cells remained viable throughout the experiment.

Activation of Macrophages with LPS

LPS-induced TNFα secretion from AM was concentration-dependent in both adhered and non-adhered groups (* $p < 0.001$). Also, AM isolated by adherence to plastic secreted significantly more TNFα in response to LPS (10 μg/ml) than non-adhered cells (314,375 ± 33,008 versus 139,826 ± 10,092 pg/ml/10^6 cells, (# $p < 0.001$)) (Figure 5.6).

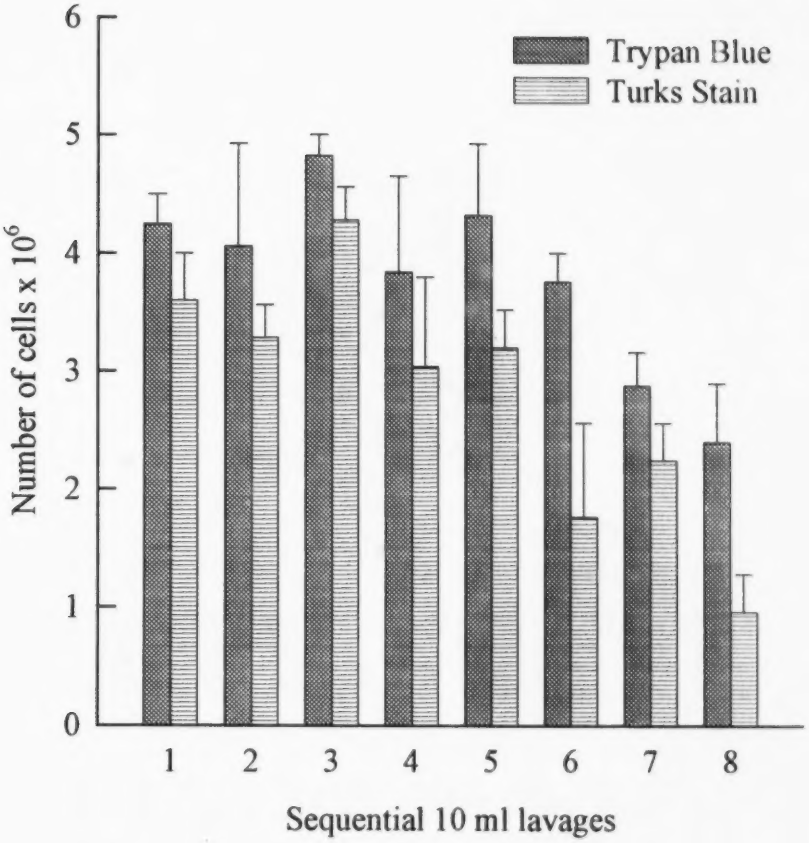

Figure 5.1. Total cell counts in combined, sequential 2 x 5 ml bronchoalveolar lavages (total volume 80 ml). Two animals were lavaged with PBS and the number of cells from each 10 ml lavage as determined by trypan blue exclusion (hatched bar) or Turks stain (lined bar). Values are the mean ± SE.

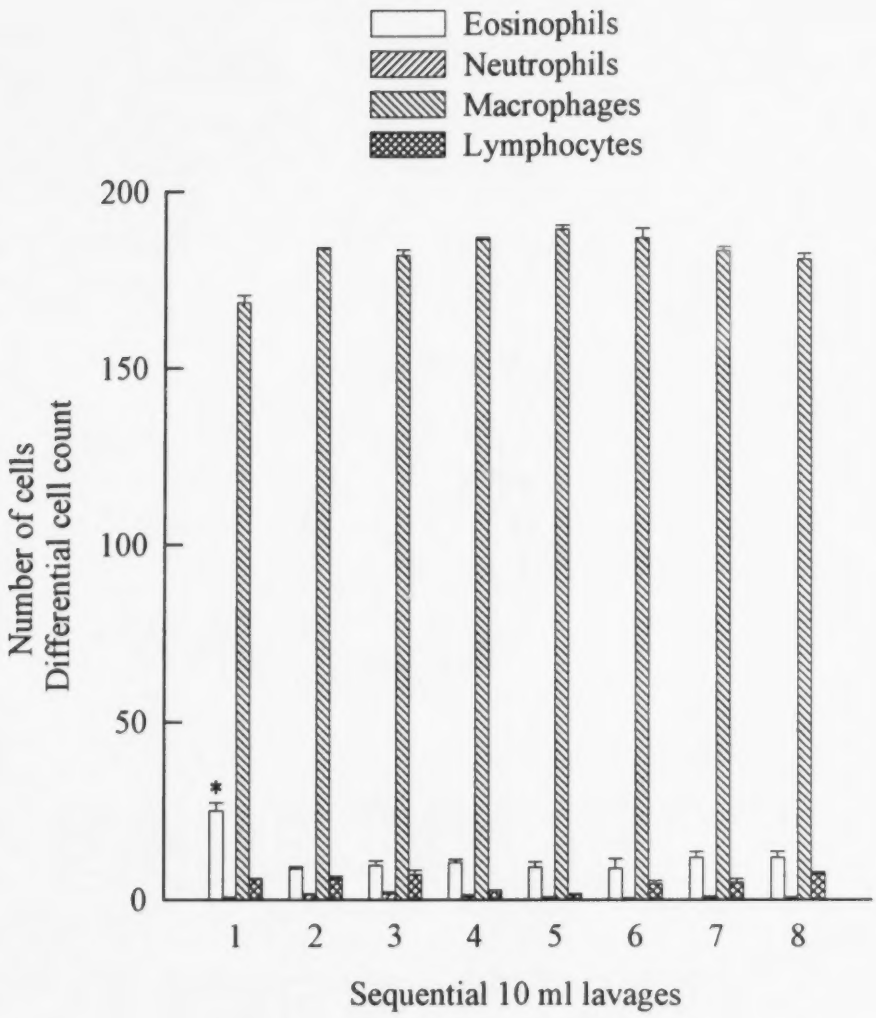

Figure 5.2. Differential cell counts in combined, sequential 2 x 5 ml bronchoalveolar lavages (total volume 80 ml). Two animals were lavaged with PBS and Cytospin slides were prepared. Differential cell counts were done on 2 x 200 cells from each animal for each 10 ml lavage aliquot. The legend is shown in the figure. * Denotes significantly higher than all other cells (except macrophages), for all lavage aliquots.

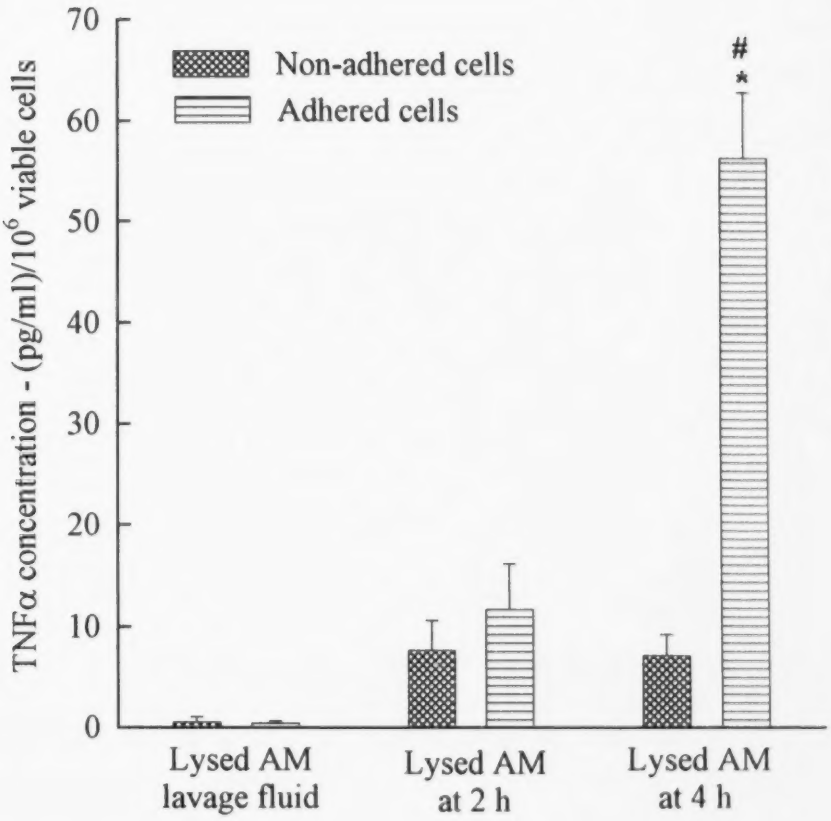

Figure 5.3. Storage of pre-formed TNFα. Two animals were lavaged and the combined AM were lysed (by freeze/thaw methods) directly from lavage fluid or incubated under non-adherent or adherent conditions, for 2 or 4 h, and then lysed. Lysates were assayed for TNFα. The legend is shown in the figure. Values are the mean ± SE. # Denotes significantly different from 2 h incubation groups. * Denotes significantly higher than non-adhered group at 4 h.

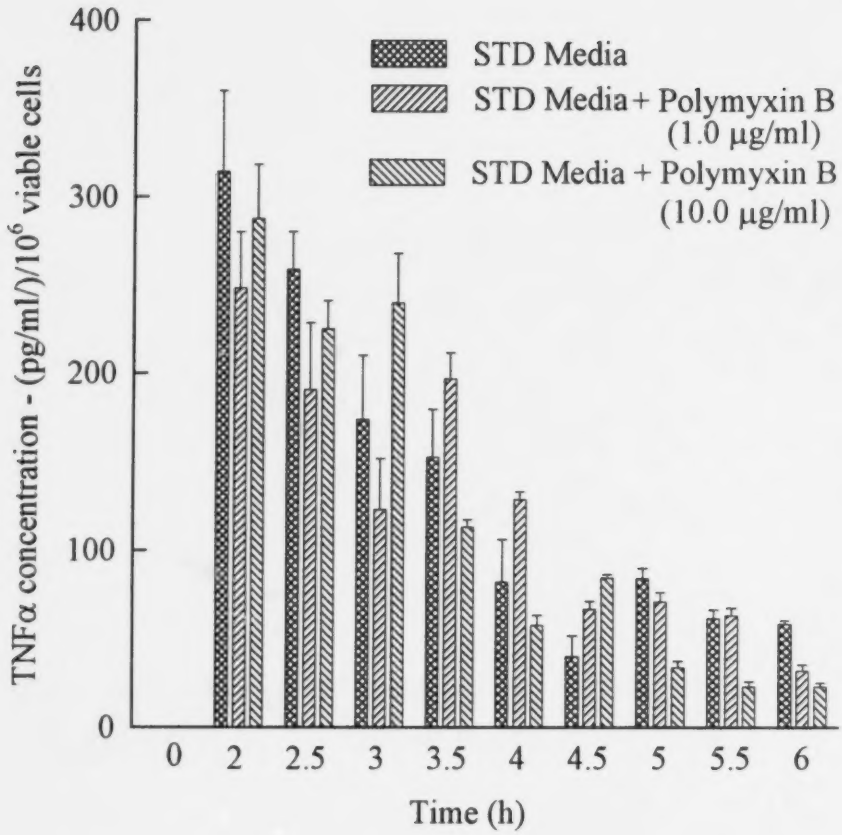

Figure 5.4. Time-dependent secretion of TNFα due to isolation of AM by adherence to plastic. Animals were lavaged and the combined AM incubated on plastic culture plates in standard medium or medium plus polymyxin B, 1.0 µg/ml, or polymyxin B, 10.0 µg/ml. Medium was collected from the lavage fluid (time 0), after a 2 h incubation, then every 30 min thereafter. Supernatants were assayed for TNFα. Values are the mean ± SE of 4 samples, assayed in duplicate. All values are significantly higher than the corresponding values in Figure 5.5.

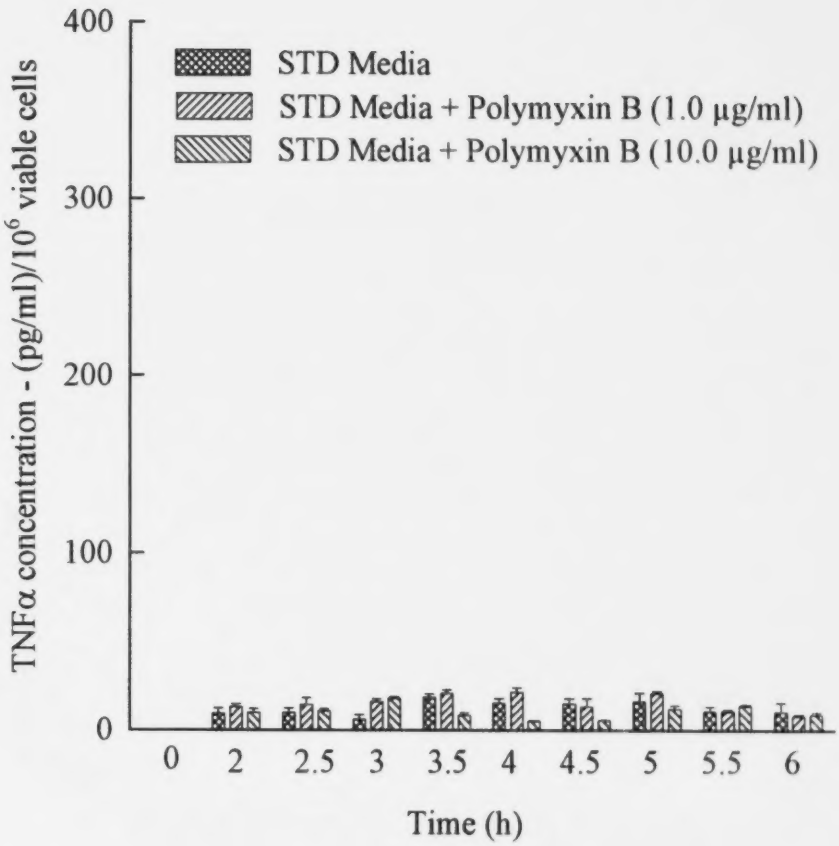

Figure 5.5. Secretion of TNFα from AM incubated under non-adherent conditions. Animals were lavaged and the combined AM incubated in 50 ml polypropylene culture tubes in standard medium or medium plus polymyxin B, 1.0 µg/ml, or polymyxin B, 10.0 µg/ml. Medium was collected from the lavage fluid (time 0), after a 2 h incubation, then every 30 min thereafter. Supernatants were assayed for TNFα. Values are the mean ± SE of 4 samples, assayed in duplicate. *All values are significantly lower than the corresponding values in Figure 5.4.*

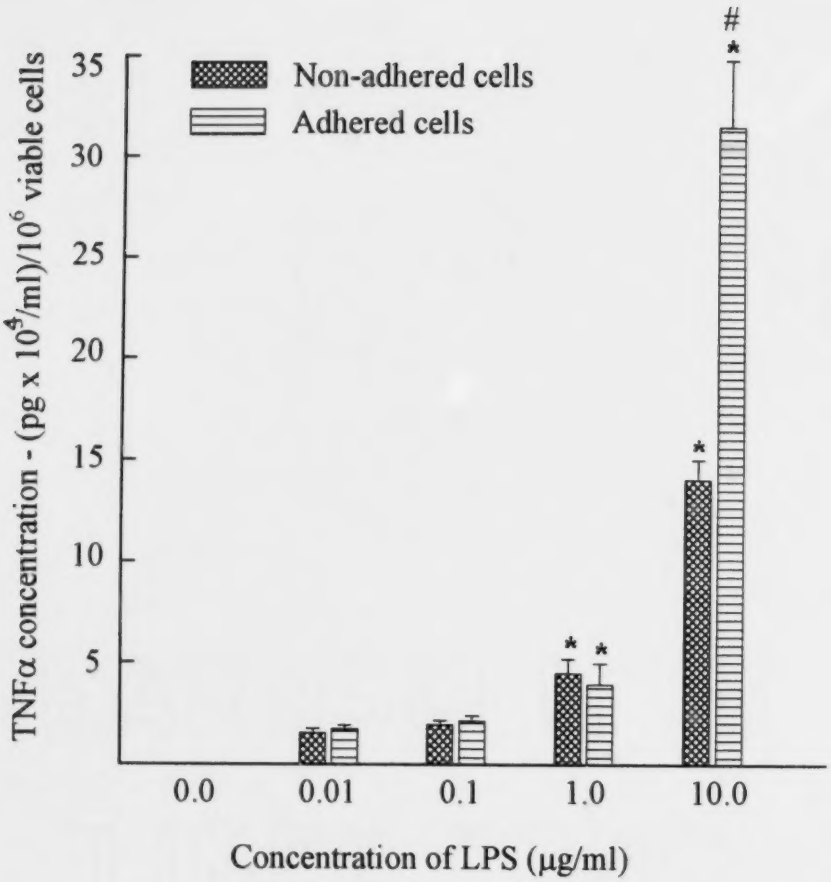

Figure 5.6. Activation of AM with LPS. Two animals were lavaged and the combined AM were activated with various concentrations of LPS after 4 h of incubation under non-adherent adherent or conditions. Supernatants were collected after 2 h and assayed for TNFα. Values are the mean ± SE of 4 samples, assayed in duplicate. * Denotes significantly higher amount of TNFα than is produced by the previous (lower) dose of LPS. # Denotes significantly higher amount of TNFα in adhered cells than non-adhered cells in response to 10.0 μg/ml of LPS.

DISCUSSION

In vivo, adherence of cells to cellular matrices is fundamental to the inflammatory response and many studies demonstrate that cells are activated by the process of adhesion to increase the message for and/or secretion of many proinflammatory polypeptides such as IL-1α, IL-1β, TNFα, CSF-1 and IL-8 (Haskill *et al.*, 1988; Fuhlbrigge *et al.*, 1987; Standiford *et al.*, 1991; Sporn *et al.*, 1990; Eierman *et al.*, 1976). These and other proteins have been implicated in the development of the inflammatory component of respiratory diseases such as asthma. Thus, inhalation of TNFα has been shown to induce airway hyperreactivity in a rat model of asthma (Kips *et al.*, 1991). TNFα has been recovered from the BAL fluid of asthmatic patients (Gosett *et al.*, 1992). Also, TNFα is believed to be involved in the process of polymorphonuclear cell recruitment during inflammatory responses (Chanez *et al.*, 1993; Wegner *et al.*, 1990). It is therefore of interest to explore TNFα secretion and its pharmacological modulation from its major cell source, the AM.

As AM are isolated routinely from other cell types by their ability to adhere to plastic cell culture plates, we have determined whether this process induces guinea-pig AM to secrete TNFα. We compared TNFα secretion from adhered and non-adhered guinea-pig AM recovered from BAL fluid. Our study showed that upon isolation of AM by a 2 hour adherence to plastic, AM secrete higher levels of TNFα, than cells that are prevented from adhering to plastic (Figures 5.4 and 5.5). After which, there is a continual decline in the concentration of TNFα detected in the supernatants collected every 30 min for a 4 h time period post adherence. Total cell counts and cell viability counts post experimentation did not change significantly, indicating that the fall in TNFα level is not due to cell death.

These results suggest that by adherence to plastic, guinea-pig AM are activated to express gene products. Haskill *et al.* (1988) showed that TNFα mRNA levels increased within 30 min of human monocyte adherence however, a second signal (LPS) was required for TNFα secretion. Standiford *et al.* (1991) demonstrated that adherence of human bronchoalveolar macrophages to plastic or cellular matrices activated this cell to express the chemotactic cytokine, IL-8, and that the translational and/or secretory efficiency of IL-8 expression was greatly enhanced by the secondary stimulus, LPS. This work also showed the upregulation of TNFα mRNA in adhered cells, however this study did not assay for the translated protein in cell supernatants. Our study demonstrates possible biochemical or phenotypical changes that macrophages undergo as a result of their isolation by adherence to plastic. Thus, adherence cannot be used as a purification method for guinea-pig alveolar macrophages used in short term assays if the cells are to be considered "non-activated".

Fluid collected by bronchial alveolar lavage has no TNFα activity, indicating that freshly isolated AM do not spontaneously secrete TNFα as a result of the lavaging process. Also, there was no TNFα in freshly harvested AM that had been lysed by freezing and thawing methods, showing that guinea-pig alveolar macrophages do not constitutively store this cytokine (Figure 5.3). Levels of TNFα detected in the supernatants from lysed cells after a 2 h adherence were minimal but increased significantly after a further 2 h incubation. However, these levels were still very low (Figure 5.3). Macrophages thus appear to secrete TNFα immediately after its synthesis and thus little is detected in supernatants from cells that have been washed 3 times post adherence, and lysed into fresh medium.

In experiments in which TNFα secretion from adhered and non-adhered cells was compared, bronchial alveolar lavage was performed on guinea-pigs with complete

medium or complete medium containing the endotoxin inhibitor, polymyxin B (1 or 10 μg/ml). AM were then isolated by adherence to plastic, or alternatively after discarding the first 10 ml of lavage fluid they were incubated under non-adherent conditions. Bacterial endotoxins are activators of macrophages (Ulich *et al.*, 1991). Thus, studies that assess cytokine secretion from macrophages must avoid endotoxin contamination. Polymyxin B binds to the lipid A portion of bacterial lipopolysaccharide and acts as a functional inactivator of LPS (Lynn & Golenbock, 1992). There were no statistically significant differences in TNFα secretion among groups of cells cultured in standard medium or medium containing polymyxin B. Also, Zhong *et al.* (1993) have recently measured the mRNA expression and protein secretion of several cytokines in differentiated human macrophages following stimulation with low doses of LPS. Their results show that levels of TNFα are measurable after stimulation with LPS at a minimum LPS concentration of 0.01 ng/ml. We used the Limulus Amebocyte Lysate Assay (Sigma) to test for endotoxin (which has a sensitivity in the picogram range) and random samples of supernatants were shown to be void of measurable endotoxin. Thus, TNFα secretion from the AM studied in our experiments is likely not arising from endotoxin contamination.

Total and differential cell counts were done on sequential 10 ml (the combination of 2 lavages of 5 ml each) lavages to determine the cellular profile. Total cell counts were done using trypan blue, which stains only non-viable cells, and Turk's stain, a cell wall stain which distinguishes nucleated cells from erythrocytes. Although not significantly different, counting total cells with trypan blue consistently gave greater total cell values than staining and counting with Turks stain (Figure 5.1). This is possibly due to the inadvertent counting of erythrocytes that may occur with trypan blue. Total cell numbers were greatest during the first 50-60 ml of lavage and tapered off with subsequent lavages. Figure 5.2 shows differential cell counts made from sequential 10

ml lavages. The greatest number of cell types other than alveolar macrophages (primarily eosinophils) was recovered in the first 10 ml of lavage. This observation is in agreement with Brunelleschi *et al.* (1990). Thus, as means to eliminate cells other than macrophages in the non-adhered cell groups, the first 10 ml of bronchial lavage was discarded, after which, cells are about 95% pure for AM.

The mechanism by which adherence induces TNFα production and secretion is not fully understood. On the cell surface of human alveolar macrophages are adhesion molecule receptors such as intercellular adhesion molecule-1 (ICAM-1 or CD54) and the integrin or CD18 molecule, LFA-1 (lymphocyte-function associated antigen-1) (Chanez *et al.*, 1993). Adhesion molecules are required for cell-to-cell interactions and thus have important roles in inflammation. They participate during the transendothelial migration of macrophage precursors (monocytes) and leukocytes from the vasculature into the alveolar space, and during antigen presentation and lymphocyte activation by macrophages. It has been reported that adherence and spreading of human monocytes to culture dishes can be blocked in the presence of the 60.3 antibody that specifically recognizes the α-chain of the LFA-1 class of receptors (Wallis *et al.*, 1985). Thus, it appears that the adherence process of macrophages to plastic is at least in part due to cellular adhesion molecules. Receptors, that are involved in interactions with other cells and those that facilitate adhesion to matrix materials are all likely to signal intracellular events that are responsible for the activation of macrophages.

Activation of cellular adhesion molecules has been reported to result in cytokine secretion. A monoclonal antibody (HP1N) which recognizes the α-chain of the LFA-1 heterodimer (CD11a) was shown to induce TNFα production from natural killer cells upon costimulation with anti-CD16 monoclonal antibody (Melero *et al.*, 1993). It could be postulated from our study that adhesion molecules present on macrophages may be

activated non-specifically to secrete the TNFα detected in the supernatants from adhered cells. A variety of signals push primed macrophages to the fully activated state. In our study, adherence not only directly induced the secretion of TNFα but further augmented LPS-induced TNFα secretion from guinea-pig AM. Our study emphasizes the need to consider phenotypical changes that cells may encounter during isolation by adherence, *in vitro*. The mechanisms by which AM activation, and TNFα secretion occurs, via adhesion to plastic requires further investigation.

CHAPTER 6

MODULATION OF TNFα SECRETION AND AIRWAYS' HYPERREACTIVITY BY THALIDOMIDE

SUMMARY

Experiments in previous chapters have demonstrated elevated levels of TNFα in the BAL fluid and from AM collected from guinea-pigs that were passively immunized with anti-OA serum, followed by challenge with antigen (OA) by inhalation. Chapter 5 outlines the refinement of our AM cell-culture model that enables investigation of TNFα production from non-adhered cells (thereby minimizing "background" TNFα production). This chapter investigates the ability of thalidomide to alter LPS-induced TNFα secretion, *in vitro*, from guinea-pig AM. We also investigate the ability of thalidomide to alter airways' hyper-responsiveness, *in vivo*, caused by histamine and LTC_4 after passive immunization and antigen challenge of guinea-pigs. The *in vitro* results show that thalidomide inhibits LPS-induced TNFα secretion from guinea-pig AM, and that the (-) enantiomer of thalidomide is more potent than the (+) enantiomer. The *in vivo* results show that the racemic mixture of thalidomide, given subcutaneously by osmotic infusion (during passive sensitization and challenge), is effective in inhibiting the airways' resistance induced by histamine in ventilated anesthetized guinea-pigs. It is possible that the decrease in airways' hyperresponsiveness is in part due to inhibition of cell-derived mediators such as TNFα by thalidomide.

INTRODUCTION

Thalidomide has been gaining favor as a drug for the treatment of graft-versus-host-disease (GVHD), leprosy, acquired immunodeficiency syndrome (AIDS) and other related diseases (Wood *et al.*, 1990; Barnes *et al.*, 1992; Sampaio *et al.*, 1991). The usefulness of thalidomide in the treatment of these diseases is ascribed to its activity as a biological response modifier. Thalidomide has been shown to prevent LPS-induced TNFα release from human peripheral blood monocytes without directly affecting either general protein synthesis or the expression of three other monocyte-derived cytokines (IL-1β, IL-6 and GM-CSF) (Sampaio *et al.*, 1991). In a subsequent study, these

authors have shown that thalidomide inhibits such TNFα production by enhancing TNFα-mRNA degradation (Moreira *et al.*, 1993). Others have shown that thalidomide has a pronounced inhibitory effect on TNFα-protein and TNFα-mRNA production in the U1 monocytic cell line activated with recombinant TNFα or with a group of other agonists, which include phytohemagglutinin (PMA), other cytokines and LPS (Madonkawkeyoon *et al.*, 1993). Thus, thalidomide has potential as an immuno-suppressive agent and is currently being investigated as an immune modulator in a number of inflammatory conditions.

The concept of asthma as an inflammatory disease has been in favor in recent years. Mast cell mediators (LTs, PGs, PAF, histamine, etc.), are released immediately from intracellular storage granules or soon after synthesis upon immunological (allergen) and non-immunological (exercise, cold air) stimuli (Lasalle *et al.*, 1991). These induce rapid edema, hyperemia and mucus release (i.e., an acute inflammatory response) followed by neutrophil and then eosinophil influx into the airways. Upon activation, macrophages participate in airway inflammation through the release of chemotactic factors (LTB_4) and cytokines (i.e., TNFα, IL-1, IL-6, IL-8, GM-CSF) (Gosset *et al.*, 1991; Burke *et al.*, 1991; Lee *et al.*, 1993). TNFα has been recovered from bronchial alveolar lavage fluid of asthmatic patients (Gosset *et al.*, 1991), and has shown to be involved in the process of leukocyte recruitment during an inflammatory response (Watson *et al.*, 1993; Wegner *et al.*, 1990). The recruitment of polymorphonuclear cells into the lungs and their activation by various stimuli, may then establish a chronic inflammatory condition. This process is thought to contribute to airway smooth muscle hyperreactivity.

We investigated thalidomide's effects on LPS-induced TNFα secretion from guinea-pig alveolar macrophages, *in vitro*. The *in vitro* studies provide data on the

racemic mixture of thalidomide as well as the optically pure enantiomers. We also determined whether thalidomide could alter the airways' hyperresponsiveness induced by agonists in an antigen-based guinea-pig model of airway hyperreactivity, *in vivo.*

METHODS AND MATERIALS

In Vitro Methods

Collection of Cells for Experiments *In Vitro*

Guinea-pigs (n = 6) were anesthetized with pentobarbital (50 mg/kg, ip). A cannula (4 cm long, PE240) was inserted into the trachea about 1 cm proximal to the larynx and an 18 G by 1.5 inch hypodermic needle inserted into the cannula and sealed in place via a short length of flexible tubing (Masterflex 96400-14). Using a 5 ml luer lock syringe, the lungs were lavaged with warm RPMI-1640 medium (i.e. standard medium (FBS 10%, L-glutamine 2 mM, penicillin/streptomycin solution 1%), by infusing the medium over 2 s, waiting 2 s, then withdrawing the medium over 2-3 s until no more could be aspirated. This procedure was repeated for a total of 16 x 5-ml lavages. The first 2 x 5 ml lavages from each animal were discarded in order to eliminate the majority of non-macrophage cell types. Fluid from each lavage was combined into 50 ml polypropylene conical tubes and stored on ice until being centrifuged (2,000 rpm, 5 min). Cell pellets were resuspended and the cells combined. Cells were counted (Neubauer hemocytometer, trypan blue exclusion stain) and adjusted to 2 x 10^6 cell/ml.

Treatment of Cells with Thalidomide

Concentrations of (±) thalidomide, (+) thalidomide and (-) thalidomide were made up fresh in standard media just prior to their incubation on cells. Cells were aliquoted (0.5 ml, i.e. 2 x 10^6 cells/ml) into 5 ml polypropylene culture tubes. One-half

ml of the racemic mixture or the enantiomers of thalidomide was added to the cells so that the final concentration of the racemic mixture of thalidomide was 0.1 to 100 mM, and that of the enantiomers was 0.05 to 50 mM. Some cells were suspended in standard medium alone (negative controls). Four samples were used for each experimental condition. The cells were incubated (37°C, 5% CO_2) for 2 h, while being constantly rocked on a plate shaker in order to minimize cellular adhesion and aggregation. LPS (*E. coli* 0111:B4, made up in standard medium) was added to the test and positive control groups to a final concentration on cells of 1.0 μg/ml. Cells were incubated for 3 h, then centrifuged (2,000 rpm, 5 min). The cell supernatants were collected and frozen until assay for TNFα using the L929 bioassay. The cells pellet was resuspended and cells were counted to determine viability post experimentation.

TNFα Measurement

As described in Chapter 5, page 92.

In Vivo Methods

Animals

As described in Chapter 2, page 46.

Preparation of α-OA-Ab Containing Serum

To obtain serum containing α-OA-IgG Ab, guinea-pigs were immunized with a single ip injection of 20 mg/kg ovalbumin (OA, grade V, Sigma) in saline. Twenty-one days later, on 8 consecutive days, animals were given pyrilamine (0.5 mg/kg, ip) and 30 min later inhaled OA (2%, in saline) aerosol (VIX Acorn nebulizer, compressed air at 15 psi) for up to 8 min. Guinea-pigs were carefully observed for the appearance of the signs of anaphylaxis. If these signs appeared, they were immediately removed from the

exposure chamber and observed. If the signs persisted, they were placed in a chamber containing 100% oxygen. If the signs still persisted, epinephrine (0.5 mg/kg) was given subcutaneously (sc). Guinea-pigs developed a predominantly anti-OA IgG-mediated reaction as defined by passive cutaneous anaphylaxis of their serum at various times post-injection and ELISA of their serum using anti-guinea-pig IgG, IgG1 and IgG2 antibodies. On the 30th day after immunization, the guinea-pigs were anesthetized with pentobarbital (40-50 mg/kg) and exsanguinated under aseptic conditions via cardiac puncture (20 ml syringe, 16 G needle). The blood was placed in a sterile glass centrifuge tubes, sealed and centrifuged at 1,500 rpm for 10 min before being stored at 4°C overnight. The tubes were centrifuged again (1,500 rpm, 10 min), the serum aspirated, pooled, placed in sterile polypropylene tubes and stored at -20°C. Serum was treated with ammonium sulfate to precipitate immunoglobulins, which were collected by centrifugation, dissolved in phosphate buffer (pH = 7.4) and dialyzed for 48 h against four changes of phosphate buffer. An aliquot of the dialyzed immunoglobulins was diluted and the titre of α-OA-Ab determined. The titre of the α-OA-Ab containing immunoglobulin solution was adjusted to a standard titre of 2,500 by addition of phosphate buffer. This standardized material was used for passive immunization (dose = 0.5 ml/kg).

Passive Immunization of Guinea-Pigs

All guinea-pigs received two injections of standardized α-OA-Ab solution (titre 1:2500) prepared as described above (0.5 ml/kg, ip), 1 d apart.

Aerosol Exposure

Each group of guinea-pigs was exposed to OA (2%) or saline aerosol for 8 min, 1 d after receiving each injection of α-OA-Ab solution. Each animal was pretreated with pyrilamine (0.5 mg/kg, ip) 30 min before inhaling either aerosol.

Measurement of Airways' Responsiveness

Guinea-pigs were anesthetized with pentobarbital sodium (35-50 mg/kg, ip). Their tracheas were cannulated and they were connected to a rodent ventilator (Ugo Basile) that provided a tidal volume of 10 ml/kg at 20 breaths/min. Air flow was measured via a pneumotach (Fleisch 0000, Validyne MP45 differential pressure transducer) and intratracheal pressure relative to atmosphere was measured via a pressure transducer (Validyne MP45). Signals were digitized and processed to yield breath-by-breath values of pulmonary flow resistance (R_L) and dynamic pulmonary elastance (E_L). A catheter was placed in a jugular vein to give drugs intravenously (iv). All animals received succinylcholine (0.1 mg/kg, iv) to prevent spontaneous breathing movements that would interfere with measurements. Increasing doses of histamine or LTC_4 were injected (iv) in increasing order of dose with recovery to baseline between injection of doses until R_L increased by at least 150% over baseline. Histamine was always given first.

Experimental Groups

Groups consisted of (A) passively immunized and challenged with normal saline in the presence of control vehicle, delivered by infusion (NS/NS); (B) passively immunized and challenged with antigen, (in the presence of control vehicle, delivered by infusion (NS/OA); (C) passively immunized and challenged with antigen (OA) in the

presence of thalidomide, delivered by infusion (THAL/OA).

Preparation of Osmotic Pumps

Sustained infusion of (±) thalidomide was achieved by employing a sterile solution in saline deployed in ALZET osmotic pumps (2 ml volume, 10 µl/h, over 7d). Sterile saline alone containing citric acid was used as control vehicle. Individual pumps were prepared for each guinea-pig. Solutions placed in the pumps were sterilized by filtration (0.22 µm syringe-end filter). Two ml of solution was placed into each pump. Pumps were primed by placing them in sterile saline at 24-26°C for 4-6 h before implantation. All procedures involved in preparation of the pumps were performed using aseptic technique. The 2ML1 pumps specifications state that they deliver 10 µl/h.

Implantation of Pumps

The ALZET osmotic pumps containing the drug or control solution were implanted sc in the nuchal region following isoflurane anesthesia using aseptic surgical technique. The procedure mimicked that shown in the pump manufacturer's instructional videotape. The incision was closed with standard wound clips. All animals received torbugesic (2 mg/kg, sc) immediately after pump implantation to control pain after surgery.

Removal of Pumps

The pump implanted in each animal was removed as soon as each guinea-pig was anesthetized for pulmonary measurements. The wound clips were removed, the incision opened and the pump removed with forceps.

Evaluation of Pump Function

The potency of the delivery portal emitting the drug solution was confirmed visually under a dissecting microscope (magnification = x 15). Then the flow moderator was removed and the fluid remaining in each pump was aspirated via a syringe and a blunt-tipped filling tube; the volume remaining in the pump was noted.

Materials

(+), (-) and (±) thalidomide (Research Biochemicals International, Natick, MA, USA); albumin, chicken egg (ovalbumin) Grade V, citric acid, leukotriene-C_4, pyrilamine maleate and succinylcholine chloride (Sigma, St. Louis, MO); Torbugesic (Sigma); histamine dihydrochloride (Fluka A.G., Buchs, Switzerland); methoxyflurane (Metophane, Janssen Pharmaceutica, Mississauga, Ont.); sodium pentobarbital (Euthanyl, M.T.C. Pharmaceuticals, Markham, Ont.). All medium and medium supplements were purchased from GIBCO BRL (Burlington, Ontario) and the cytokine research products from Genzyme Diagnostics (Cambridge, MA, USA).

Statistical Analyses and Data Expression

Differences among groups were analyzed using Student's "t" test, one-way ANOVA, Kruskul-Wallis and Dunn's one-way ANOVA on ranks, and multiple comparison tests (Student-Newman-Keuls Test). SigmaStat was used to apply these tests. Significance was assumed at the 5% level. TNFα concentration values obtained from the bioassay are expressed as percent of control (with background values subtracted) and are plotted against the log 10 concentration of thalidomide.

RESULTS

In Vitro Experiments

Inhibition of LPS-Induced TNFα Production from AM

Figure 6.1 represents a plot of the log 10 concentration of thalidomide ((±), (+) or (-)) versus TNFα concentration (background TNFα concentrations are subtracted), expressed as percent change from control. These data are the combined values of two experiments (i.e. for each experiment, the cells from 6 animals were combined and 4 samples of each experimental condition were then evaluated in duplicate for TNFα levels). Thalidomide and its enantiomers are effective in inhibiting LPS-induced TNFα secretion from guinea-pig AM, in a dose-dependent fashion. The (±) compound provided about 42% inhibition of LPS-induced TNFα secretion at 0.1 μM, and 65% inhibition at 100 μM (the highest concentration examined). The (-) enantiomer showed a 20% inhibition at a 0.05 μM concentration and this increased to about 59% inhibition at 50 μM. The (+) compound, at a concentration of 0.05 μM, was ineffective at inhibiting LPS-induced TNFα secretion, but provided about 44% inhibition at a concentration of 50 μM. Thus, comparing the two optically pure enantiomers, (-) thalidomide was more effective that (+) thalidomide at inhibiting LPS-induced TNFα secretion.

In Vivo Experiments

Evaluation of Pump Function

Inspection of the emitting orifices of the pumps showed all to be patent and apparently functioning properly. Aspiration of the remaining solution in each pump revealed that less solution had been delivered to each animal than anticipated. Each pump should have delivered about 1.6 ml of solution (10 μl/h x 24 h x 7d), but only about

1.2 ml, approximately 72% of the amount predicted, was delivered. There was no statistically significant differences among the pump volumes delivered among the three experimental groups.

Baseline Measurements of R_L and E_L

There were no statistically significant differences among the baseline measurements of R_L and E_L. Data are summarized in Figure 6.2. All animals were passively immunized with α-OA-Ab containing serum. Control groups were infused with control vehicle and aerosoled with saline or OA (NS/NS or NS/OA, respectively). The test group was infused with thalidomide and aerosoled with OA (THAL/OA).

Dose-Response Curves to Histamine and LTC_4

Changes in R_L and E_L in response to graded doses of histamine and LTC_4 (both iv) in the three groups are shown in Figures 6.3, 6.4, 6.5 and 6.6. We first compared dose-response curves to histamine in guinea-pigs that had received α-OA serum (ip) and inhaled saline (Group A, NS/NS)) or inhaled OA (2%) aerosol (Group B, NS/OA); in both groups the osmotic pumps contained control solution. For changes in R_L induced by histamine (iv), inhalation of OA aerosol induced about a two-fold, statistically significant ($p < 0.05$) shift of the dose-response curve to the left, in comparison with inhalation of saline aerosol. Only at high dose, (1.0 μg/kg) was a similar shift noted when LTC_4 was the agonist. Similar findings were noted with measurements of changes in E_L only in response to high dose histamine (10.0 μg/kg) ($p < 0.05$) and high dose LTC_4 (1.0 μg/kg) ($p < 0.1$).

We compared dose-response curves to histamine and LTC_4 in guinea-pigs in Groups B and C. Both groups received α-OA serum (ip); both groups inhaled OA aerosols. In Group B, the pump contained control solution; for Group C, it contained

(±) thalidomide. In our guinea-pig model of asthma, thalidomide (25 mg/kg/d) given by osmotic pump prevented increased airways responsiveness to histamine ($p < 0.05$, Figure 6.3), but not to LTC_4.

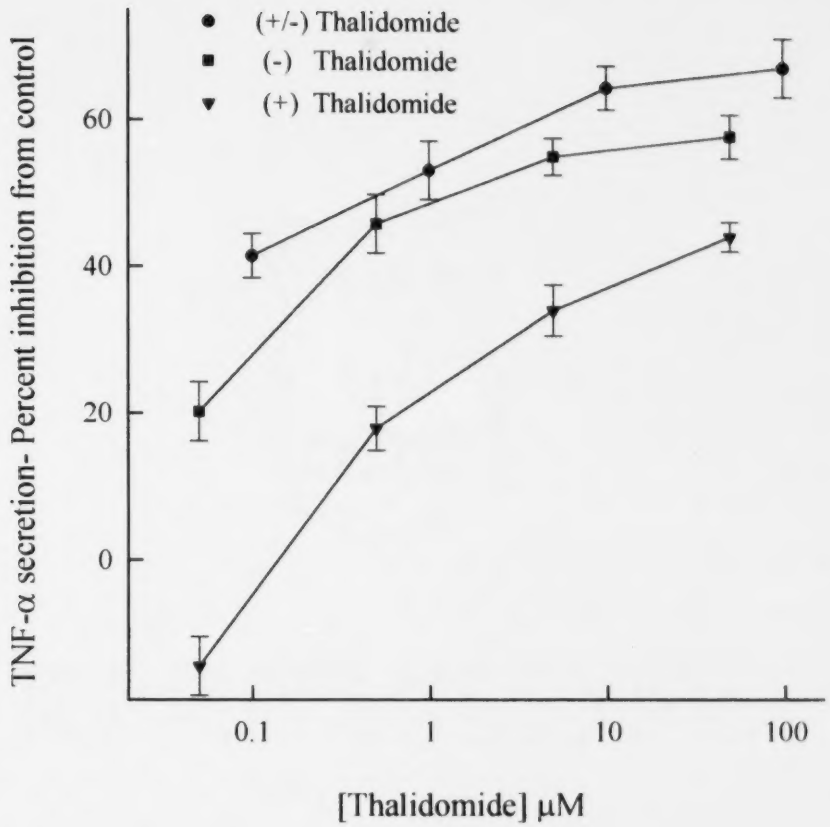

Figure 6.1. TNFα secretion (with background subtracted and expressed as percent change from control) versus the log10 concentration of thalidomide. Guinea-pig AM were incubated with (±), (+), or (-) thalidomide for 2 h, then stimulated with LPS (final concentration on cells 1.0 mg/ml) for 3 h. Supernatants were collected and assayed for TNFα using the L929 bioassay. These data are the combined values of two experiments (i.e. for each experiment, the cells from 6 animals were combined and 4 samples of each experimental condition were then evaluated in duplicate for TNFα levels).

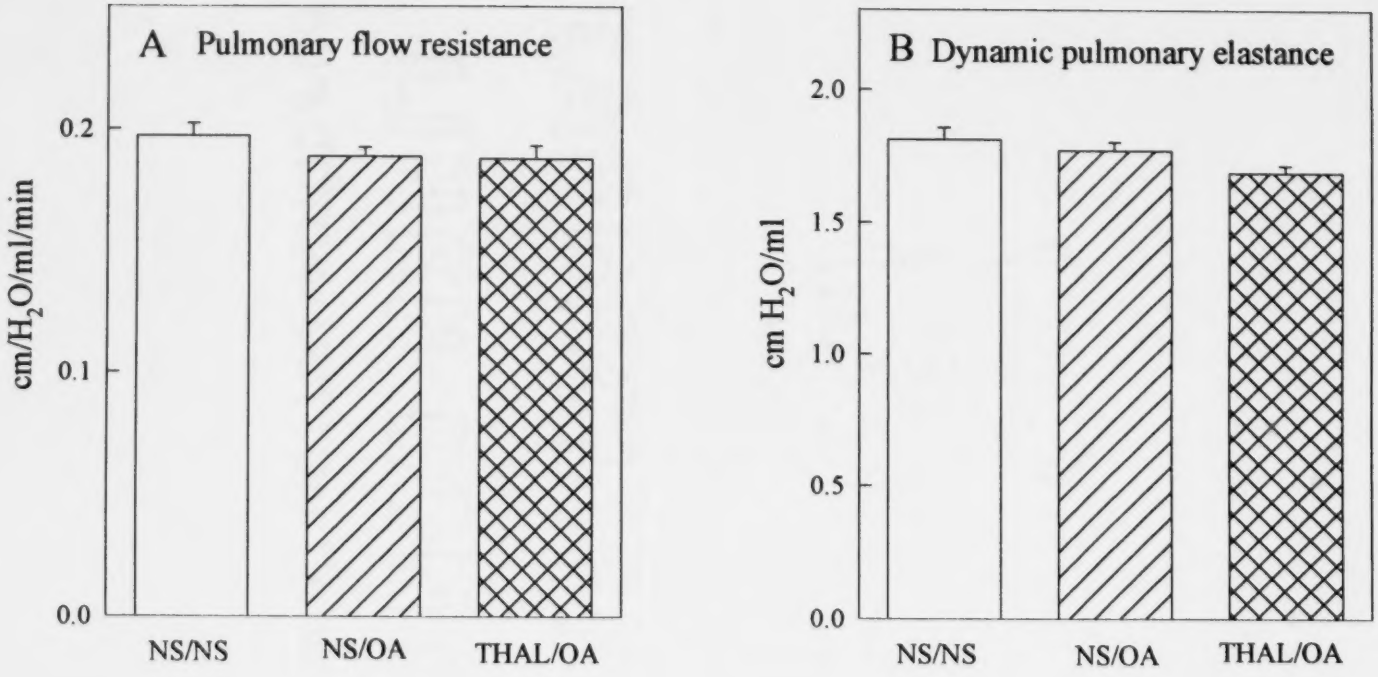

Figure 6.2. Baseline values for resistance (A) and elastance (B). Animals were all passively immunized with α-OA-Ab. Open bar group received saline by infusion, and saline aerosol. Forward-slash bar group received saline by infusion, and antigen (OA) aerosol. Double-hatched bar group received thalidomide by infusion and antigen (OA) aerosol. There were no statistically significant differences in the values among the group ($p > 0.05$).

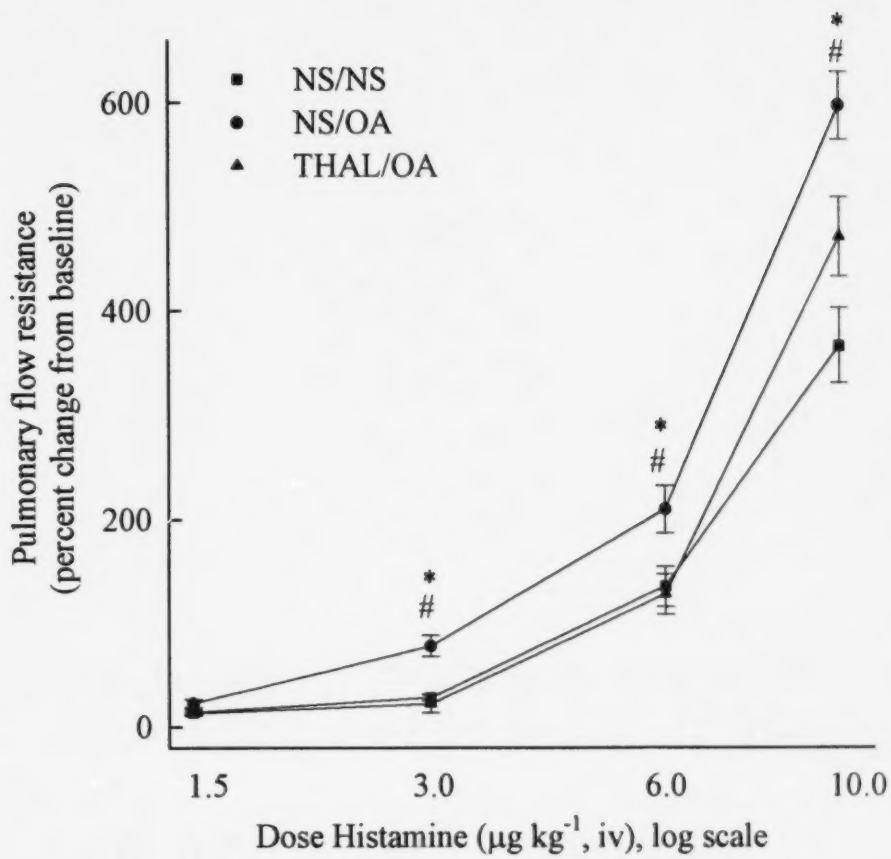

Figure 6.3. Changes as percent of baseline values, in pulmonary flow resistance in anaesthetized, paralyzed guinea-pigs following injection (iv) of histamine in control and test groups. All animals were passively immunized with α-OA-Ab containing serum. Control groups were infused with control vehicle, and aerosoled with saline or OA (NS/NS or NS/OA, respectively). The test group was infused with thalidomide and aerosoled with OA (THAL/OA). Each point represents the mean ± SEM of 5 or 10 animals. # Denotes significantly different in NS/OA group compared to NS/NS group. * Denotes significantly different in THAL/OA group compared to NS/OA group.

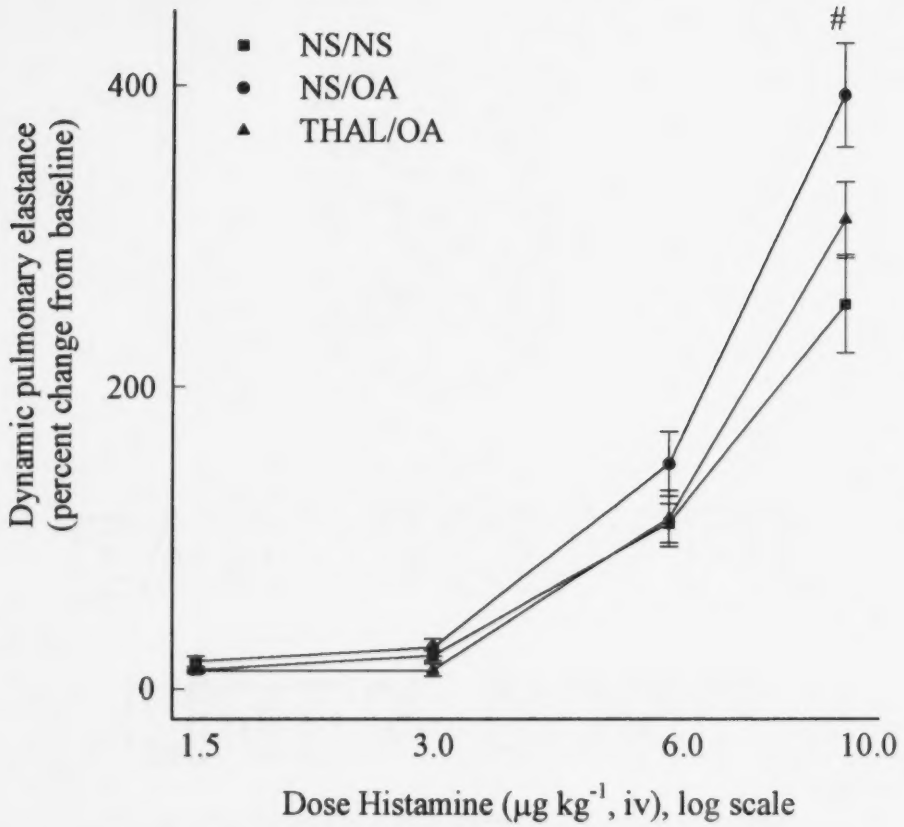

Figure 6.4. Changes as percent of baseline values, in dynamic pulmonary elastance in anaesthetized, paralyzed guinea-pigs following injection (iv) of histamine in control and test groups (groups described in Figure 6.2). Each point represents the mean ± SEM of 5 or 10 animals. # Denotes significantly different in NS/OA group compared to NS/NS group.

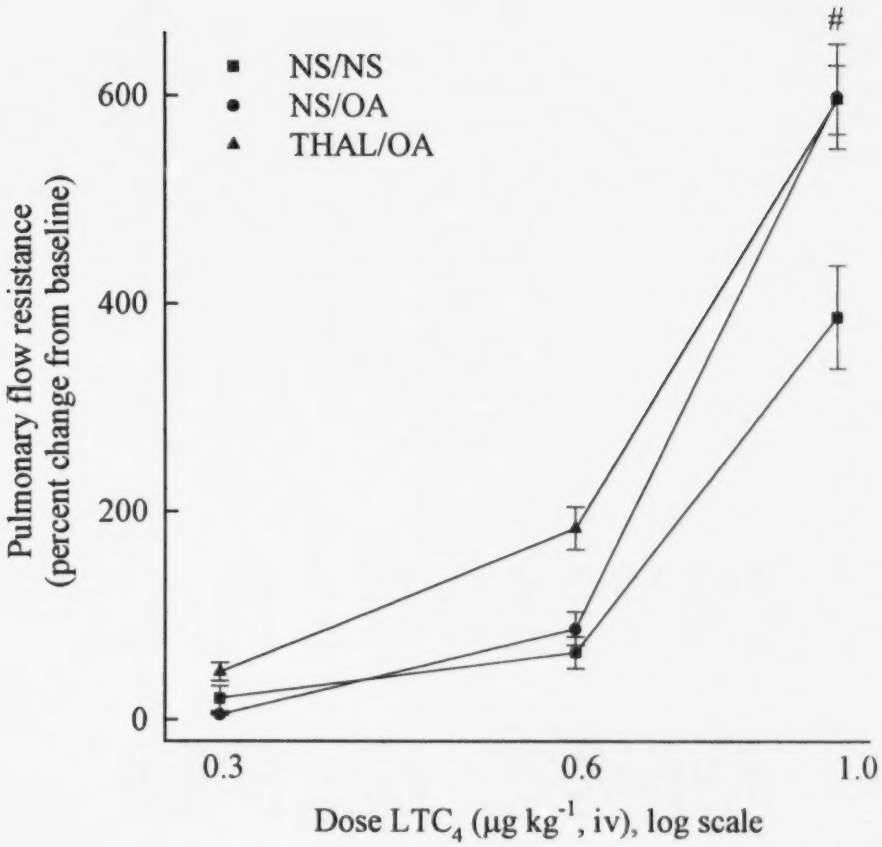

Figure 6.5. Changes, as percent of baseline values, in pulmonary flow resistance in anaesthetized, paralyzed guinea-pigs following injection (iv) of LTC_4 in control and test groups (groups described in Figure 6.2). Each point represents the mean ± SEM of 5 or 10 experiments. # Denotes significantly different in NS/OA group compared to NS/NS group.

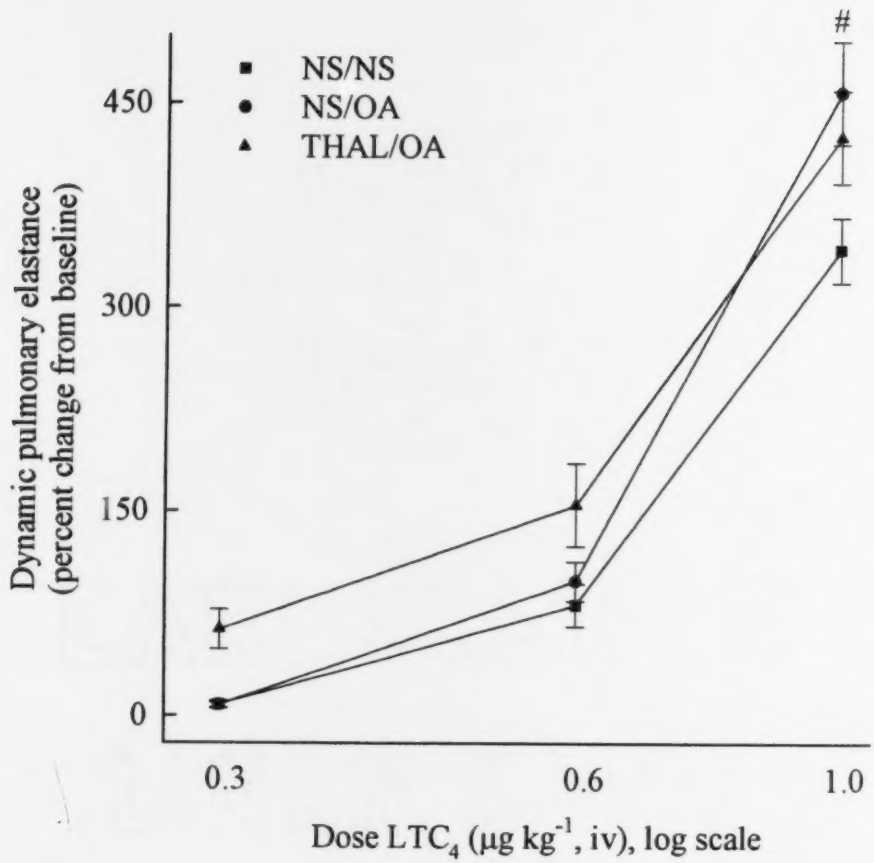

Figure 6.6. Changes, as percent of baseline values, in dynamic pulmonary elastance in anaesthetized, paralyzed guinea-pigs following injection (iv) of LTC_4 in control and test groups (groups described in Figure 6.2). Each point represents the mean ± SEM of 5 or 10 experiments. # Denotes significantly different in NS/OA group compared to NS/NS group.

DISCUSSION

In the present study we investigated the modulation of LPS-induced TNFα secretion from guinea-pig AM by the racemic mixture and both of the optical enantiomers of thalidomide. In light of these experiments, we then evaluated whether thalidomide affects the *in vivo* airway hyperreactivity induced by LTC_4 and histamine in ventilated anesthetized guinea-pigs that have been passively immunized with α-OA antiserum and then challenged with antigen to induce airways' hyperresponsiveness.

From the *in vitro* studies, we report a dose-dependent inhibition of LPS-induced TNFα secretion by the racemic mixture of thalidomide and for each of the optical enantiomers; the (-) enantiomers displays more potency at TNFα inhibition than the (+) enantiomer. Cell viability remained constant throughout the experiment (data not shown) indicating that changes in TNFα concentration were not due to cell death caused by thalidomide. As well, thalidomide, at the highest concentrations used in experiment, was shown not to have effects on the L929 cell line used in the TNFα detection assay. Results from Moreira *et al.* (1993), suggest that the inhibition of LPS-induced TNFα production from peripheral blood monocytes by thalidomide, can be equally achieved by both of the enantiomers (their data was not shown). However, in those experiments, the enantiomer compounds were incubated with the cells and agonist (LPS) for a total of 20 h. This time course needs to be taken into consideration in light of a recent publication examining the rate of racemization of the optically pure forms of thalidomide. Half completion time of racemization for the (-) enantiomers of thalidomide in PBS (at pH 7.4, 37°C), is 9.43 h (Nishimura *et al.*, 1994). At 20 h, therefore, the amount of pure enantiomers left in solution is low. Thus, in investigating the biological activity of each pure optical enantiomers, it is of paramount importance to use fresh solutions of compound as well as to keep the experimentation time to a minimum. Our thalidomide

compounds were in solution for a total time of approximately 5 h. We chose this time period for two reasons; 1) our preliminary studies indicated that after a 3 h incubation in the presence of (±) thalidomide and LPS, TNFα production was significantly inhibited from control (approximately 43%), and 2) the enantiomers would likely not entirely convert over this relatively short time period. Racemization of the enantiomers however, may still help to explain why the results do not entirely support the absolute biological activity of one enantiomer over the other. Using methylthalidomide prepared as the non-racemizable (+) and (-) analogs of thalidomide, Nishimura *et al.* (1994) demonstrated that the (-) form is biologically active at modulating TNFα production in their *in vitro* experiments. Thus, the racemization of the pure optical enantiomers of thalidomide needs to be taken into consideration in experiments evaluating the molecular mechanisms of thalidomide's action.

Although we did not evaluate the secretion of other cytokines in these experiments, thalidomide is reported to be specific in its inhibition of LPS-induced TNFα secretion from human monocytes; the amounts of IL-1β, IL-6 and GM-CSF remained unaltered (Sampaio *et al.*, 1991). Our findings are in accordance with studies that report thalidomides' *inhibition* of LPS-induced TNFα secretion from human monocytes (Madonkawkeyoon *et al.*, 1993; Sampaio *et al.*, 1992). It must be noted, however, that other investigators report thalidomide-induced *enhancement* of TNFα secretion post stimuli. Thalidomide enhanced TPA-induced production of TNFα by human leukemia cell lines including HL-60, K562 and U937 (which is a macrophage-like cell line) (Nishimura *et al.*, 1994). Differences in thalidomide activity may suggest differences in the molecular mechanisms of TPA versus LPS action and/or thalidomide in different cell types.

In our *in vivo* experiments, thalidomide, infused subcutaneously by osmotic pump during the sensitization and challenge procedure, inhibited the increase in airways' responsiveness induced by histamine in this antigen model of airway hyperreactivity. This is shown by thalidomide's significant inhibition of histamine-induced increases in pulmonary resistance in antigen challenged animals ($p < 0.05$, Figure 6.3). This is not observed for histamine-induced changes in pulmonary elastance, likely due to the degree of variation in these measurements and also the lower sensitivity of this parameter. When LTC_4 is the agonist, the enhancement of airway hyperreactivity in challenged versus non-challenged animals (as shown by an increase in percent change in resistance and elastance) is achieved only at the highest LTC_4 dose, and thalidomide does not alter this effect.

A more complete knowledge of thalidomide's biological effects is currently being sought. In evaluating thalidomide for the treatment of erythema nodosom leprosum (ENL), thalidomide therapy reduced not only serum TNFα levels but also the dermal infiltration of polymorphonuclear leukocytes (PMN) and T-lymphocytes (Sampaio *et al.*, 1993). The expression of ICAM-1 and MHC class II antigens on epidermal keratinocytes was also down-regulated in the presence of thalidomide. In support of thalidomide effects on surface adhesion molecules, a study in monkeys demonstrated thalidomide-induced alterations in surface adhesion receptors (LFA-2, LFA-1α, LFA-1β) on T- and B-lymphocytes, monocytes and neutrophils (Neubert *et al.*, 1993). In humans, thalidomide demonstrated simultaneous up- and down-regulation of different integrin receptors on white blood cells, *in vivo* (Neubert *et al.*, 1992; Nogueira *et al.*, 1994). Also in the treatment of ENL, thalidomide has been show to cause a decrease in the number of $CD4^+$ T-lymphocytes in the blood (Shannon *et al.*, 1992), and to inhibit lymphocyte proliferation *in vitro* (Keenan *et al.*, 1991). In mice, a single treatment with thalidomide, depleted $CD4^+$ and $CD8^+$ cells in the thymus and lymphoid cells in the bone

marrow (Arala-Chaves *et al.*, 1994). Thus, thalidomide may inhibit T-lymphocyte and PMN recruitment to inflammatory sites by regulation of cellular adhesion molecules, or alternatively, by affecting the life-span or proliferation of T-cells and PMN.

In the context of our interest, which is airway disease, airway inflammation in asthma is described as a complex multicellular event, leading to morphological changes that may be the driving force in the development of bronchial hyperresponsiveness (Busse & Reed, 1993; Reid *et al.*, 1989). Numerous studies have identified the early response cytokines, IL-1 and TNFα, in the initiation of inflammatory cell adherence to the vascular endothelium by initiating the expression of cellular adhesion molecules (reviewed in Fiers, 1991; Camussi *et al.*, 1991; Tracey, 1994). Thus, TNFα release by activated macrophages or T-lymphocytes is a regulatory step to initiating cellular recruitment and inflammation. Although in our system, the precise *in vivo* biological and molecular effects of thalidomide remain to be determined, it could be postulated that by inhibiting cytokine (i.e. TNFα) production from a number of cell types (discussed previously), thalidomide may suppress inflammation due to alterations in the cellular recruitment process following antigen challange.

Recent research in the field of asthma has been to evaluate the usefulness of specific phosphodiesterase (isotype-IV) inhibitors for the treatment of airway hyper-responsiveness and inflammation. One such compound, pentoxyfylline, has been shown, *in vitro*, to suppress the synthesis of TNFα, and *in vivo*, to protect experimental animals against endotoxic shock (the single most important mediator in endotoxic shock is known to be TNFα) (Zabel *et al.*, 1993). Thalidomide has been shown to suppress TNFα production in monocytes by enhancing TNFα-mRNA degradation (Moreira *et al.*, 1993). By comparison, pentoxifylline, a methylxanthine derivative, reduces TNFα-mRNA accumulation by inhibiting transcription of the TNFα gene (Doherty *et al.*,

1991), while the glucocorticoid, dexamethasone, inhibits TNFα production primarily post-transcriptionally by reducing translation (Han *et al.*, 1990). Thus, it is conceivable that pentoxyfylline or glucocorticoids could provide additive effects on TNFα inhibition when used in combination with thalidomide.

CHAPTER 7

PRODUCTION OF A HETEROHYBRIDOMA THAT SECRETES OVALBUMIN SPECIFIC MONOCLONAL ANTIBODY FOR THE PURPOSES OF *IN VITRO* ACTIVATION OF GUINEA-PIG ALVEOLAR MACROPHAGES

SUMMARY

We hypothesized that AM may be activated to secrete TNFα via cross-linking of Fcγ receptor-bound IgG (and thus "cross-linking" of Fcγ receptors) by addition of antigen. To further investigate this hypothesis, we attempted to produce a guinea-pig/murine or guinea-pig/rat heterohybridoma that secretes anti-OA monoclonal antibody of a guinea-pig IgG class. This antibody would then be used *in vitro*, to delineate mechanisms of IgG-induced TNFα secretion by guinea-pig AM. Also, it would allow refinement of the *in vivo* passive sensitization model by replacing the polyclonal anti-serum presently used, with a monoclonal antibody. In these experiments intra-species fusions were successful as demonstrated by the production of antigen and class specific guinea-pig antibody (shown by ELISA). However, clone stability was poor. Antibody secretion could not be maintained for sufficient periods of time to allow for recloning and purification of antibody. Thus, as an alternative, anti-OA IgG polyclonal antibody was purified from the serum of actively immunized guinea-pigs (described in Chapter 8).

INTRODUCTION

In order to investigate the mechanisms that govern Fcγ receptor activation of cells it would be beneficial to have in large amounts, an ovalbumin specific monoclonal antibody of an IgG isotype. This would allow refinement of ongoing experiments to evaluate antigen specific activation of cells via the Fcγ receptor since most current work of this nature in guinea-pigs is possible only with pAb. Having a mAb (of known isotype) rather than a pAb (which is a mixture of Ig classes and isotypes) for *in vitro* and *in vivo* passive sensitization would allow for more control over the antibody response, and thus provide more specific information about the response. Another advantage of

hybridoma-derived Ig is the relative purity and ease of preparation of the mAb. In this study, we sought to produce a monoclonal antibody that recognized hen egg ovalbumin (OA) and was of a guinea-pig IgG class.

Methods for the production of such antibody secreting cell lines were first described by Kohler and Milstein (1975). This technique relies on the development of myeloma cell lines that grow in normal culture medium but not in a defined selection medium because they lack a functional salvage pathway gene required for DNA synthesis in this selection medium. There are two pathways of purine synthesis that exist in cells; the *de novo* pathway and the salvage pathway. Aminopterin is a drug used in selection medium that blocks the normal *de novo* pathway and shifts the cell into using the salvage pathway for purine synthesis. The salvage pathway however, requires external sources of thymidine and/or hypoxanthine for the production of nucleotides, and the cell uses the enzymes thymidine kinase (TK) and/or hypoxanthine guanine phosphoribosyl transferase (HGPRT) for the synthesis of nucleotides. In hybridomas, the necessary genes required for DNA synthesis are thus supplied from the normal B cells allowing only the somatic hybrids to grow in the selection medium. So, hybridoma cells like normal cells may grow normally in the presence of aminopterin if the culture medium is also supplemented with thymidine and hypoxanthine (called HAT medium). The cell lines used as fusion partners are rendered deficient of TK- or HGPRT- by selection mutagenesis, and thus do not have the genetic machinery to survive in HAT medium unless fused to a normal cell such as a B cell. If such normal cells are fused to TK- or HGPRT-negative cells, the normal cells provide the necessary enzymes so that the fused hybrids synthesis DNA and grow in HAT medium. Moreover, genes from the myeloma cell render such hybrids immortal. Normal B cells, on the other hand, generally do not survive for a long time in culture and die in culture within 10 to 14 days (described in more detail in Cellular and Molecular Immunology, Abbas AK, Lichtman AH, Pober JS,

eds). Such fusion-derived immortalized antibody-producing cell lines are called hybridomas and the antibodies they produce are monoclonal antibodies.

The fusion of a myeloma cell with a splenic B cell from two different species results in a heterohybridoma. This form of monoclonal antibody production is often chosen when good intraspecies fusion partners are unavailable, and such is the case for guinea-pig. In our studies, well established murine (SP2/0) and rat (Y0) myeloma lines were chosen as fusion partners.

Our results show that successful fusions between both guinea-pig B cells and rat myeloma, and guinea-pig B cells and murine myeloma cell lines were produced. However, the limitation to their further "work up" and use in *in vitro* experiments was the inability of these heterohybrids to maintain long term antibody secretion. It is well known that interspecific somatic hybrids preferentially lose chromosomes from one of the fusion partners, usually leading to loss in ability to secrete the immunoglobulin of interest (Sogn, 1987).

METHODS

Immunization Schedule

Guinea-pigs were immunized using several different immunization protocols in an attempt to produce primarily an IgG antibody response:

Protocol 1

Animals were immunized with hen egg ovalbumin (OA, grade iv, Sigma), (20 mg/kg, ip) on day 1. On days 21 to 28 inclusive, they received aerosols of OA (2% in normal saline) for up to 4 min each day. On day 35, they were sacrificed and their spleens excised for collection of B cells. For one of the fusions, the retropharyngeal, the

internal iliac and the mesenteric and colonic lymph nodes were also excised as a source of B cells used for fusion.

Protocol 2

Animals were immunized with OA (20 μg/kg mixed with 10 mg/kg $Al(OH)_3$, ip) on day 1. On day 14 they were given an intrasplenic injection/challenge of OA (total dose 60 μg). The intrasplenic injection was performed as previously described (Spitz, 1986). Briefly, the animal was anesthetized with isoflurane, and an oblique incision was made on the left side of the abdomen, above the spleen. Using forceps, the spleen was lifted from the abdominal cavity and injected with OA solution (0.5 ml volume, 60 μg OA in normal saline). The spleen was replaced, the incision sutured and the animal allowed to recover. On day 18 the animals were sacrificed and their spleens excised for the collection of B cells.

Protocol 3

Animals were immunized with OA (20 mg/kg, ip) on day 1. On day 14 they were given an intrasplenic injection/challenge of OA (total dose 60 μg). The intrasplenic injection was performed as described above, and the spleen excised on day 18.

Fusion of Guinea-Pig B Cells to Murine or Rat Myeloma Cells

The cell lines chosen as fusion partners were the rat Y0 myeloma cell line, and the murine SP 2/0 myeloma cell line (both obtained from ATCC). The fusion of splenic or nodal B cells to these myelomas was achieved using either PEG or electrofusion techniques.

(i) *Methods for PEG Fusion*

B cells were flushed from the spleen or lymph nodes with warm medium using a 5 ml syringe and a 28 G needle. These cells were collected and mixed with either SP2/0 or Y0 cells in approximately a 1:5 ratio of myeloma to B cells. The combined cells were washed twice with warm medium and the final wash was aspirated from the cell pellet. Polyethylene glycol (PEG, 0.5 ml, MW = 1500, Sigma) was carefully added to the cells, drop by drop. The tube containing the cells was centrifuged for a total of 8 min, after which the cells were carefully resuspended in 5 ml of medium with a final addition of another 5 ml of medium containing 20% FBS. At this stage, the cells were only minimally disturbed. The cells plus medium were centrifuged for 5 min and washed twice with the addition of medium. The supernatant was aspirated and the cells were resuspended in medium containing 20% FBS, 1% penicillin/streptomycin solution, 1% glutamine, 1% oxalic acid-pyruvate-insulin (OPI) and 1% HAT. The cells were plated into 96-well culture plates at approximately 1.5×10^5 cells/well (in 200 µl) and incubated for 5-7 days. Medium was then aspirated and replaced with fresh HAT medium and clones were left to grow prior to screening by ELISA.

(ii) *Methods for Electrofusion*

A solution of sterile 0.3 M glucose plus 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$ was prepared (pH adjusted to 7.3 with 0.1 N NaOH). Spleen or nodal cells were collected and combined with the fusion partner myeloma cell line at a 1:4 myeloma/lymphocyte ratio, in standard medium. The mixture was centrifuged, the supernatant was aspirated from the cell pellet and the cells then washed a total of 3 times with the sterile glucose solution. The cells were resuspended to a final concentration in the glucose solution of approximately 1×10^6 cells/ml for fusion. The settings on the ECM 200 electrocell manipulator (BTX Inc., San Diego, CA), were adjusted while observing the alignment

of cells under the microscope. They were found optimal when using the Electrode BTX microslide chamber 450 (0.5 mm gap, chamber volume 20 µl), at:

Alignment Amplitude:	5 V
Alignment Time:	30 Sec
Desired Field Strength	4.5 V
Electrofusion Amplitude	100 V
Pulse Width	30 usec
Number of Pulses	1

The microslide was sterilized in 70% ethanol prior to placement on the microscope stage. The micrograbber cables were attached to the (+) and (-) posts on the microslide. Cells (20 µl) were carefully pipetted between the microslide bars. The ECM200 was activated to deliver first the alignment AC pulse, followed by the electrofusion pulse. The cell suspension was then removed (under sterile conditions) from the fusion chamber and placed into a sterile culture tube. Standard medium containing antibiotics, glutamine, 20% FBS and 10% Origen (Gibco) was added and the cells incubated at 36°C for 15 min. Cells were then centrifuged (500 rpm, 5 min) and resuspended in enough HAT medium so that they were plated into 96-well culture plates at a concentration of from 200 to 1000 cells per well in 200 µl. The cells were incubated, the medium was aspirated on the following day and the cells were re-fed with fresh HAT medium. They continued to be incubated for 10 to 14 days in order to form colonies of hybrid cells.

Post Fusion Hybridoma Culture Maintenance

HAT medium was aspirated and replaced from the cells approximately 5-7 days post fusion. Once visible colonies were apparent, 100 µl of supernatant was collected

from such wells for antibody screening. Cells from those wells testing positive for OA or guinea-pig IgG antibody specificity were transferred to 24-well culture plates. Additional HAT medium was added to these cells over time until the cells had multiplied to confluence. The supernatant was then retested by ELISA for the desired antibody. Those colonies testing positive were grown up in 6-well culture plates and the supernatants screened again for antibody. A limiting dilution was done on several of the positive clones, and the 96-well plates screened again for antibody.

Screening of Hybridoma Supernatant for Antibody by ELISA

Enzyme linked immunosorbent assay (ELISA) techniques were used to detect guinea-pig IgG, and to then detect ovalbumin specific antibody. Two assays were employed and are outlined in the following step charts.

ELISA 1: is a sandwich ELISA that detects IgG antibody.

1. Immunosorbent ELISA plates (Nunc Immunosorbant plates, Maxisorb, Gibco, Burlington, Ontario, Canada) were coated with anti-guinea-pig IgG antiserum (developed in goat, Sigma) (75 µl of a 1:10,000 dilution) and left to bind overnight.

2. The plates were emptied and 1% BSA or skim milk (200 µl) was added to block unbound sites. Plates were incubated overnight in the refrigerator, or for 3 h at 36°C.

3. Plates were washed with a 0.02% Tween 20 in PBS solution and samples of hybrid supernatants were added (100 µl). Plates were incubated overnight in the refrigerator, or for 3 h at 36°C.

4. Plates were washed and the second (detection) antibody added was an HRPO labeled anti-guinea-pig IgG, developed in goat (Sigma), (100 µl of a 1:10,000 dilution).

The plates were incubated for 1 h at 36°C.

5. Plates were washed 3 times and blotted dry by inverting onto a paper towel. Enzyme-substrate was added (ABTS/H_2O_2, 50 µl, Kirkeggard and Perry Laboratories Inc., Gaithersberg, Maryland) and the colour developed over 25 to 30 min.

6. Optical densities were read at 405 nm, using an automated plate reader and titers estimated using SOFTmax Software, Version 2.01d, Molecular Devices.

Serum containing polyclonal anti-ovalbumin IgG that was developed in actively immunized guinea-pigs was used as a positive control. Medium without antibody or supernatants from non-ovalbumin specific hybridomas were used as negative controls.

ELISA 2: is a sandwich ELISA that detects ovalbumin specific antibody of an IgG class.

1. The protocol is identical to that previously described, except that the ELISA plates were coated with ovalbumin (20 µg/well in 75 µl) rather than anti-guinea-pig IgG antiserum. This ELISA is thus not specific only for IgG, but detects ovalbumin specific antibodies of the IgG class.

RESULTS

Antibody Secretion from Heterohybridomas

Table 7.1 summarizes the data from the various fusion procedures employed. The immunization protocol used, the fusion method chosen and the number of positive clones at the various stages of culture are shown.

Briefly, several observations regarding these fusion experiments are notable:

1. The fusion efficiency was generally low.

2. The morphology of some antibody-secereting hybrids was unusual. Some clones that tested positive for secretion of desired antibody had "fibroblast-like" characteristics (see Figure 7.2). These cells were adherent and had focal development of cells that then spread out across the bottom of the culture plate. Around the periphery of these focal developments, budding-off round cells (that were more typical of hybrid morphology) were observed (compare Figure 7.1 with 7.2, 7.3 and 7.4). Other colonies resembled typical, round, non-adherent "hybridoma-like" cells (see Figure 7.1), some of which secreted the desired antibody.

3. The secretion of desired antibody from the colonies fell consistently as the colonies were transferred from 96- to 24- to 6-well culture plates, until such time that antibody secretion from all fusions was absent or negligible.

TABLE 7.1

Fusion Number	Immunization Protocol	Fusion Partner	Fusion Method	Total Clones	IgG Clones			α-OA-IgG Clones		
					96 well	24 well	6 well	96 well	24 well	6 well
P 69	1	SP 2/0	PEG	6	4	3	0	0	-	-
P 70	2	SP 2/0	PEG	35	22	9	0	10	4	0
P 71	2	YO	PEG	0	-	-	-	-	-	-
P 72	3	SP 2/0	PEG	26	10	5	0	12	5	3→0
P 73	3	YO	PEG	67	35	12	0	28	13	3→0
P 80	2	SP 2/0	ELECTRO	0	-	-	-	-	-	-
P 85	2	SP 2/0	PEG	18	-	-	-	0	-	-
P 86	2	SP 2/0	ELECTRO	27	7	4	0	0	-	-
P 92	1	SP 2/0	PEG	41	8	6	0	3	2	0
P 93	1	SP 2/0	PEG	45	12	8	0	4	1	0
P 94	1	SP 2/0	PEG	15	8	3	0	2	2	0
P 95	1 (lymph nodes)	SP 2/0	PEG	0	-	-	-	-	-	-

Summary of data from heterohybrids. Guinea-pig B cells were fused with either murine SP2/0 or rat Y0 myeloma cell lines. The fusion method employed is indicated (PEG or electrofusion (ELECTRO)). The total number of clones produced from each fusion is given, and the number of those clones that were IgG-isotype specific are indicated (from the original 96 well plates, then the 24 and 6 well transfers). Clones which were OA specific are also indicated.

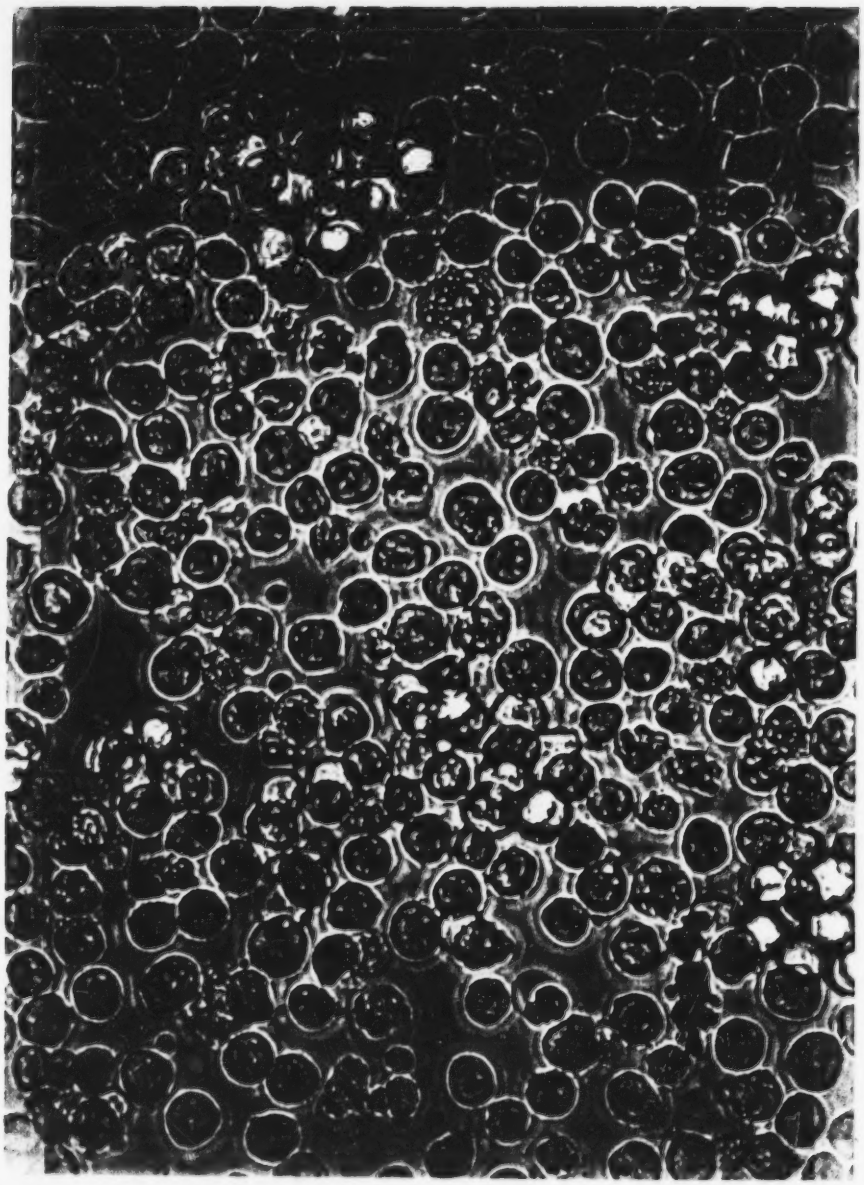

Figure 7.1. Photograph of normal, typical heterohybridoma fusion. Cells are round, uniform and non-adherent. Microscope magnification 500 X.

Figure 7.2. Photograph of an atypical heterohybridoma fusion colony capable of antibody secretion. Cells are fibroblast-like, adherent, and grow in patches. Round, non-adherent cells appear to bud off from the focal development. Microscope magnification 500 X.

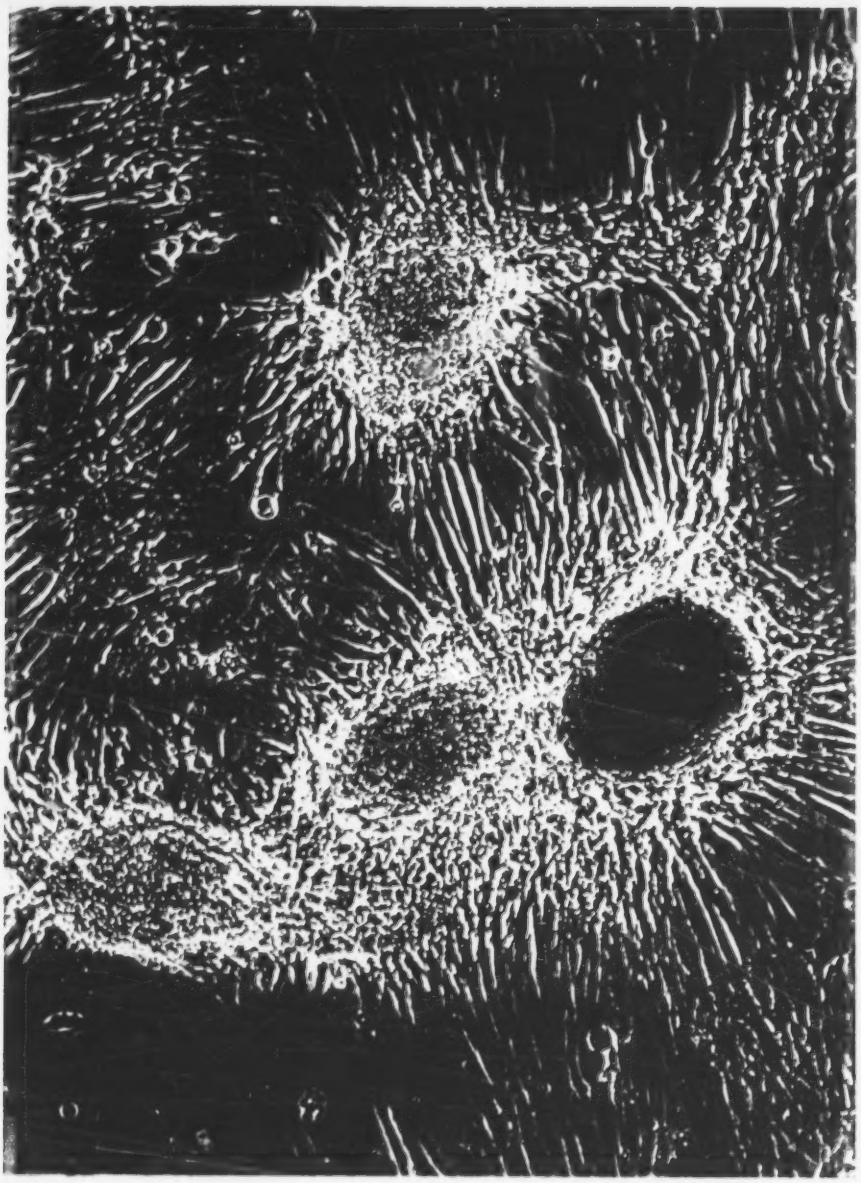

Figure 7.3. Phase-contrast photograph of atypical heterohybridoma fusion colonies capable of antibody secretion. Note the fibroblast-like characteristics. Microscope magnification 250 X.

Figure 7.4. Photograph of an atypical heterohybridoma fusion colony capable of antibody secretion. Cells are fibroblast-like, adherent, and grow out from focal developments such as that shown here. Round, non-adherent cells appear to bud off this adherent focal cell mass. Microscope magnification 1000 X.

DISCUSSION

To date, no guinea-pig hybridomas or heterohybridomas reportedly exist. We attempted to fuse guinea-pig B cells with rat Y0 or murine SP2/0 myeloma cell lines. Both of these interspecies heterohybridoma pairs (i.e. guinea-pig and rat, and guinea-pig and murine) appear to be genetically unstable. Our data shows that successful fusions were obtained (as shown by positive ELISA results for the antibody of interest), however, secretion of the desired antibody was not maintained by these hybrids for any longer than approximately 3 weeks. This is a common limitation to the production of most heterohybridomas. Rabbit-mouse hybrids, for instance, are very unstable with respect to secretion of the rabbit immunoglobulin and human-mouse hybrids are intermediate in their rate and extent of chromosomal loss when compared to other species pairs (Sogn, 1987).

The production of immunoglobulin in a B cell requires a complex process of gene rearrangement. Genes encoding the two light chains (κ and λ) and the single locus containing the various heavy chain genes are located on different chromosomes (detailed Golub & Green, 1991). In humans, the H chain locus is on chromosome 14, the κ light chain locus on chromosome 2 and the λ light chain locus on chromosome 22. Thus, secretion of antibody requires at least the retention of two chromosomes from the somatic parent cell of interest (i.e. to give rise to one heavy chain and one light chain). The process is complex however, and other factors are involved. Non-secreting human-mouse hybrids, shown to contain the necessary antibody chromosomes were stimulated to secrete immunoglobulin with mitogen (LPS) (Raison *et al.*, 1982). Thus, the cessation of immunoglobulin secretion may be caused by factors other than loss of structural genes. In our studies, some clones were cultured in the presence of IL-2 (to promote immunoglobulin production) in an unsuccessful attempt to maintain antibody

secretion (data not shown).

Immunization protocol methods, fusion partners and fusion protocols were varied in our experiments to try and optimize conditions for continued antibody secretion. Further, "back-fusions" between the newly developed non-secreting murine/guinea-pig hybrids and normal guinea-pig B cells were also attempted (data not shown). The selection process in this case becomes difficult as the murine/guinea-pig fusion partner has the ability to grow in the HAT selection medium as well as standard medium. Thus, this fusion was done using electrofusion methods and plating the cells at a limiting dilution (i.e., 1 cell/well). Unfortunately no antibody secreting hybrids were detected.

Ideally, a guinea-pig myeloma cell line needs be developed with a sensitivity to HAT medium (i.e. HGPRT-) such that fusion and selection processes with normal guinea-pig B cells can be possible. Then, culture conditions for maintenance of antibody secreting hybrids needs to be optimized. As our attempts to develop a guinea-pig anti-ovalbumin mAb were unsuccessful future, experiments involved the purification of antigen specific antibody from the serum of actively immunized guinea-pigs for use in preliminary *in vitro* experiments as elaborated in the next chapter.

CHAPTER 8

DOES CROSS-LINKING OF Fcγ-RECEPTORS VIA BOUND IgG PLUS SOLUBLE ANTI-IgG-ANTIBODY OR ANTIGEN INDUCE TNFα PRODUCTION FROM GUINEA-PIG ALVEOLAR MACROPHAGES?

SUMMARY

In addition to generating IgE antibodies in an allergic response, IgG antibodies are produced. Thus, it is possible that part of the allergic reaction involves the activation of cells by IgG as well as IgE. These experiments are designed to evaluate IgG-induced TNFα secretion by guinea-pig AM, and thus define a possible role for IgG in hypersensitivity reactions. Using labelled (i.e. with ^{125}I) and unlabelled guinea-pig IgG in a competitive binding assay, the association constant (K_a) for monomeric whole IgG to guinea-pig AM was shown to be 1.24×10^9 M^{-1}. As well, the number of binding sites per cell was calculated to be 1.54×10^5. Thus, in our system we demonstrate high affinity binding of guinea-pig IgG to guinea-pig AM. Next, polyclonal anti-OA IgG antibody was purified from the serum of actively immunized guinea-pigs by affinity chromatography. Studies then evaluated the effect of purified cell-bound antigen-specific IgG plus soluble antigen, to induce TNFα production from guinea-pig AM, *in vitro*. Parallel experiments evaluated whether AM could be activated by cross-bridging of cell-bound whole IgG by polyclonal or monoclonal anti-guinea-pig IgG antibody, to produce TNFα. To prime AM for enhanced TNFα production (and thus detection), the three experimental conditions were performed in the presence and absence of IFNγ. Results show that none of these conditions produced amounts of TNFα above that observed in control groups alone. Experiments evaluating TNFα production by *preformed* antigen-antibody complexes, may provide useful information about possible mechanisms of IgG-induced TNFα production in hypersensitivity reactions.

INTRODUCTION

Mast cells are traditionally recognized as the initial effector cell leading to the generation of the early asthmatic response. They release an array of inflammatory and spasmogenic mediators (i.e. histamine, tryptase, prostaglandins and cytokines) upon cross-linkage of membrane anchored Fcε receptors by interactions with membrane-

bound antigen specific IgE plus inhaled antigen. In allergic diseases such as asthma, antigen-specific IgG antibodies are produced in addition to IgE antibodies. Thus, in mechanisms analogous to IgE antibodies, IgG antibodies may also have the capacity to activate cells to release inflammatory and spasmogenic mediators.

Tumor necrosis factor alpha (TNFα) is released from mast cells via IgE receptor cross-linkage, and much literature supports the involvement of this cytokine in the pathology of inflammatory diseases such as asthma (described in previous thesis chapters). Another cell source for TNFα is the alveolar macrophage (AM). AM products such as PAF, LTB_4, GM-CSF, and TNF have the capacity to recruit and activate other inflammatory cells, leading to the perpetuation of the inflammatory response. The AM is the predominant cell type in the airways and is likely exposed to antigen upon inhalation. We have previously shown that after antigen administration (by inhalation) to passively immunized guinea-pigs, TNFα is detectable in the lavage fluid as soon as between 30 and 60 min post antigen challenge. Also in this model, AM retrieved by lavage are upregulated to secrete this cytokine (Kelly *et al.*, 1992; Denis *et al.*, 1994). The antibody used in these experiments for passive immunization was of an IgG class.

Studies have reported the release of TNFα in rat lungs and from rat AM via IgG and IgG receptor (i.e. FcγR) activation. In an *in vivo* IgA immune-complex lung injury model, IgG immune complex (i.e. rabbit IgG-anti BSA plus BSA) administration resulted in a marked rise in intra-pulmonary TNFα activity. It was accompanied by progressive pulmonary PMN accumulation (Warren *et al.*, 1991). Also in this study, exposure of isolated AM to preformed IgG-immune complexes resulted in dose-dependent TNFα secretion. Taken together, this information provides a rational for investigating mechanisms of IgG-induced TNFα secretion from AM, *in vitro*. In this study, we first determine the binding affinity and the number of binding sites (i.e. Fcγ

receptors) for whole IgG to guinea-pig AM. We then investigate the ability of macrophage-bound guinea-pig IgG plus soluble anti-guinea pig IgG monoclonal antibody (mAb) or soluble anti-guinea-pig IgG polyclonal antibody (pAb) to induce TNFα from guinea-pig AM. Also, bound, purified guinea-pig anti-ovalbumin IgG polyclonal antibody (α-OA-pAb) plus soluble antigen (OA) is assessed for its ability to induce TNFα production from guinea-pig AM . These three experimental groups are evaluated both in the presence and absence of IFNγ, a known activator of AM.

MATERIALS AND METHODS

Development of Ovalbumin Specific Guinea-Pig Serum

As described in Chapter 2, page 48.

Passive Cutaneous Anaphylaxis (PCA) to Measure IgE and IgG Antibodies

Methods used for PCA are described (Karol *et al.*, 1991). Briefly, guinea-pigs were anesthetized with pentobarbital (20-30 mg/kg, ip) and their backs shaven. A commercial depilatory was used to further remove remaining hair. Animals were allowed to recover and after 24 h they were again anesthetized and dilutions of guinea-pig sera (that known to contain IgE as a positive control, and the sera being presently tested) were injected intradermally within sections of a grid marked on the animals back. Animals were allowed to recover and after 2 or 7 d, the animals were anesthetized, the right jugular vein isolated and cannulated and a solution of Evan's Blue Dye (2 % in normal saline) and ovalbumin (0.1 %) was injected, iv, over about 1 min. After 30 min the animals were killed with an overdose of pentobarbital, then skinned. The diameter of the areas showing extravasation of dye on the inside surface of the skin were measured. A rating scale was used to relate the activity of the diluted sera to the size of the PCA

response. In guinea-pigs, IgE can be distinguished from other homocytotropic antibodies by heating the serum for 4 h at 56°C (which destroys IgE activity) or by waiting >5 d post injection of serum before challenging (IgE are long-term sensitizing antibodies whereas IgG are short-term) (Karol *et al.*, 1991).

Purification of Anti-ovalbumin Guinea-Pig Immunoglobulins

Approximately 120 ml of whole guinea-pig serum was collected. The serum was centrifuged to remove cellular debris and diluted 1:1 with phosphate buffered saline, pH = 7.4 (PBS). Ammonium sulfate was added to the serum/PBS mixture to achieve a 45% (v/v) saturated solution of ammonium sulfate. This was left to stir overnight at 4°C to ensure precipitation of all protein. The mixture was then centrifuged (3000 rpm, 30 min), the supernatant removed and the precipitate dissolved in a minimum volume of PBS. The dissolved precipitate was dialyzed against PBS for 48 h at 4°C (changing the dialysis buffer 4 times), after which the volume of the sample was approximately 60 ml.

(i) Thiophilic Gel Chromatography

T-Gel, a thiophilic gel was purchased from Pierce Chemical Co. (Rockford, IL, USA) and manufacturer's instructions were followed for the purification of immunoglobulin. Briefly, potassium sulfate was added to the sample so that the final concentration was 0.5 M potassium sulfate. This was centrifuged, the supernatant collected and filtered, then applied to the 20 ml T-Gel column that had been equilibrated with the binding buffer (0.5 M potassium sulfate, 50 mM sodium phosphate, 0.05% sodium azide, pH 8.0). The column was washed with binding buffer to remove unbound material. The absorbance (A_{280}) was monitored until baseline was achieved. The immunoglobulin was eluted with the elution buffer (50 mM sodium phosphate, 0.05% sodium azide, pH 7.4). The protein absorbance was monitored and the eluted

peak collected as a whole fraction. The column was regenerated by washing with 8 M guanidine hydrochloride. The column was rinsed with several bed volumes of deionized water followed by a bed volume wash of storage buffer (0.5 M Tris, 0.05% sodium azide, pH 7.4), and the column allowed to drain. Storage buffer was added to the column, and the column refrigerated until further use.

This procedure was repeated 4 times until all the serum had been processed. The purified serum was then dialysed against PBS. Samples were then concentrated by centrifugation using 30 Centri-preps (Amicon Co., Beverly, MA, USA) and SDS-PAGE electrophoresis (PhastGel IEF System, Pharmacia, Baie d'Urfé, Québec) was done to assess purity of the bound fraction.

(ii) Ovalbumin Sepharose Affinity Chromatography

The OA specific antibody was then purified by affinity chromatography. For the preparation of a 20 ml OA affinity column, 200 mg of hen egg OA (Grade V, Sigma) was dialyzed overnight in binding buffer (0.5 M sodium phosphate, pH 7.5). Cyanogen bromide activated Sepharose 4B beads (4 g, Sigma) were allowed to swell in the binding buffer (1 h with gentle stirring). The activated beads were added to a 50 ml glass column and washed 10 times the bed volume with binding buffer (under vacuum). The beads were removed from the column, mixed with the OA solution and incubated overnight at 4°C, with gentle stirring to allow the beads to couple the OA molecule. The OA-cyanogen bromide coupled beads were returned to the column and washed twice the bed volume with the binding buffer. This wash was saved and the A_{280} to assess the efficiency of OA binding to the column. The column was washed once with 1 M sodium chloride in 0.05 M sodium phosphate, (pH 7.5). The beads were again removed from the column and incubated for 4 h with 10 bed volumes of 100 mM ethanolamine in 0.5 M sodium phosphate (pH 7.5), with gentle mixing. The beads were returned to the

column, washed twice with PBS and stored refrigerated in PBS (plus 0.02% sodium azide) until use.

The prepared column was washed with 20 bed volumes of PBS. A peristaltic pump was connected to the column and T-Gel bound-fraction eluates were passed through the beads at a flow rate of approximately 2 ml/hr. The protein A_{280} was monitored while the unbound fractions were washed from the column with binding buffer and baseline was achieved. The column was then washed with 10 bed volumes of pre-elution buffer (10 mM phosphate buffer, pH 6.8), to allow for a quicker change to the elution conditions. While monitoring absorbance, the elution buffer (100 mM glycine, pH 2.5) was passed through the column and the eluted peak collected into the neutralizing buffer (1 M phosphate, pH 8.0). After which, the column was regenerated by passing 20 bed volumes of PBS through the matrix and stored refrigerated in PBS (plus 0.02% sodium azide). The bound fractions were combined and dialysed against PBS (48 h with 4 buffer changes), and an SDS-PAGE electrophoresis (Phast Gel IEF System) was done to assess purity. The sample was concentrated by centrifugation in 30 ml Centri-preps.

Calculation of the Extinction Coefficient for Whole Guinea-Pig IgG

Solutions of various concentrations (mg/ml) of whole guinea-pig IgG (Sigma, lyophilized, not less than 95% pure as shown by SDS-Page (reduced)), were made in PBS. After subtracting the A_{280} of PBS (background), the A_{280} of the IgG solutions were measured. The data plotted as a straight line (Excel) and the value for the slope of the line was used as the extinction coefficient for guinea-pig IgG.

Iodination of Guinea-Pig IgG

A stock solution of IODO-GEN ((1,3,4,6-tetrachloro-3,6-diphenylglycouril, Sigma) was prepared by dissolving 2 mg in 10 ml of chloroform. Fifty µl was then added to a small glass culture tube and evaporated with a stream of air (leaving approximately 10 µg or reagent plated onto the walls of the reaction vessel). Lyopholized guinea-pig IgG (Sigma) was solubilized in PBS (10 mg/ml) and 100 µl (1 mg) was added to the reaction vessel. 200 µl of 0.05 M phosphate buffer was added to ^{125}I-NaI (2 mCi, Amersham) and 50 µl of this (500 µCi) was added to the reaction vessel. The electrophilic species formed in the solid state reaction between iodine and IODO-GEN then undergoes an electrophilic aromatic substitution with one or more of the aromatic protons in the tyrosine amino acid groups of the dissolved protein. This reaction is allowed to proceed for 30 min with occasional mixing. The reaction mixture was then transferred to a clean vessel and 20 µl of freshly prepared 1 M NaI was added to the reaction mixture and left for 10 min.

To remove free radioiodine, gel column exclusion was done. A pre-packed 5 ml Sephadex PD-10 column (Pierce) was washed with 1% BSA in PBS. The radioactive protein mixture was added to the column and eluted with 0.05 M PBS and collected into 40 separate vials (approximately 8 drops/fraction). Each tube was counted in a sodium iodide detector. The higher molecular weight of the labeled protein allows it to pass freely through the column while the lower molecular weight material is retained longer. Thus the fractions from the first peak of activity are representative of the labeled protein and were kept and combined. The amount of protein in solution was measured by reading the absorbance at 280 (A_{280}).

Competitive Binding Assay on AM

Bronchoalveolar lavage was performed on four guinea-pigs as described below. The fluids were centrifuged and the cells resuspended in standard medium. The cells were plated into 96-well culture plates (5×10^5 cells/well in 300 µl) and incubated for 2 h to allow for macrophage adherence to plastic. The supernatants were aspirated and the cells washed once with warm medium. Labeled protein (0.01 µg in 100 µl) was added to each well (excluding a control group) and various concentrations of unlabelled guinea-pig IgG was added to each well (samples were done in triplicate, and the assay performed in sterile PBS plus 0.5% BSA and 0.2% sodium azide, pH 7.3). The final reaction volume was therefore 200 µl. The samples were incubated at 36°C for 30 min, then at 4°C for 3 h. At the end if incubation, 150 µl of supernatant was drawn from each well and the radioactivity measured in a gamma counter. Analysis was performed using GraFit software.

Collection of AM for IgG/Antibody or Antigen Activation Studies

Groups of normal guinea-pigs were anesthetized with pentobarbital (50 mg/kg, ip). Their tracheas were dissected free and cannulated with a 4 cm long piece of PE240 tubing that had inserted within it an 18 G by 1.5 inch hypodermic needle. The needle was sealed in place via a short length of flexible tubing. Using a 5-ml luer lock syringe, the lungs were carefully lavaged with warm standard medium (RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin solution). A total of 80 ml (i.e. 16 x 5 ml lavages) was instilled into each animal. Fluids were collected, combined and centrifuged. The cell pellets were resuspended with small amounts of medium and all the cells combined. The concentration of cells was adjusted to 1×10^6 cell/ml and 1 ml plated into each well of 24-culture well plates. Cells were left incubated for 2 h in order for macrophages to adhere to the plastic, after which the non-adherent cells were

then washed away with warm medium.

Description of Experimental Groups for IgG Activation Studies

Cells were incubated for 30 min at 36°C, then overnight at 4°C, in standard supplemented RPMI-1640 medium (Gibco) alone or medium that contained whole guinea-pig IgG (150 μg/ml, 1 ml (Sigma)) or the purified anti-ovalbumin guinea-pig IgG (150 μg/ml, 1 ml). The amount of IgG used was determined from the previous binding analysis and was chosen to be approximately 100 x Kd or 150 μg/ml (1 ml). Experiments were performed on the following day and experimental groups are outlined in Table 8.1.

Part A, describes the control groups. To assess background secretion of TNFα, cells (1 x 10^6/well) were incubated in standard medium alone (1 ml), or medium that contained the individual compounds used for study (i.e. whole IgG, antigen specific IgG, the anti-IgG antibodies, ovalbumin, IFNγ).

Part B, describes the anti-guinea-pig IgG polyclonal antibody (pAb) test group. Cells were incubated in standard medium containing whole guinea-pig IgG (150 μg/ml, 1 ml), for 30 min at 36°C, then overnight at 4°C. The medium was aspirated and replaced with standard medium that contained anti-guinea-pig IgG polyclonal antiserum (developed in goat, Sigma) at various dilutions (1:100, 1:500, 1:100, or 1:2000). The cells were incubated for 6 h at 36°C after which the supernatants were collected and frozen (-80°C), until assay for TNFα.

Part C, describes the anti-guinea-pig IgG monoclonal antibody (mAb) test group. Cells were incubated in standard medium containing whole guinea-pig IgG (150 μg/ml, 1 ml), for 30 min a 36°C, then overnight at 4°C. The medium was aspirated and replaced with standard medium that contained anti-guinea-pig IgG murine monoclonal antibody

(grown in ascites fluid, Sigma) at various dilutions (1:100, 1:500, 1:100, or 1:2000). The cells were incubated for 6 h at 36°C after which the supernatants were collected and frozen (-80°C), until assay for TNFα.

Part D, describes the purified anti-ovalbumin guinea-pig polyclonal IgG antibody (α-OA-pAb) plus antigen (OA) test group. Cells were incubated in standard medium containing purified anti-ovalbumin guinea-pig polyclonal IgG antibody (150 μg/ml, 1 ml), for 30 min at 36°C, then overnight at 4°C. The medium was aspirated and replaced with standard medium containing ovalbumin antigen at various molar concentrations of ovalbumin to anti-ovalbumin antibody (0.25, 0.5, 1.0, 2.0). The cells were incubated for 6 h at 36°C after which the supernatants were collected and frozen (-80°C), until assay for TNFα.

In all test groups, the 6 h activation step was performed with and without recombinant-murine IFNγ (1.0 U/ml) to assess the ability of IFNγ to prime AM for enhanced TNFα production.

All three experimental conditions were first performed individually, in the presence of appropriate controls. Concentrations of compounds, numbers of cells (and thus animals required) as well as endotoxin detection and removal were studied and optimized. The data shown here are from one large experiment performed during one time period, and are consistent with data from the initial studies. In this study, eight guinea-pigs were lavaged and the cells combined. Macrophages were selected for by adherence, and four samples of each experimental condition (i.e. each concentration of compound) were established. IgG activation experiments were then carried out and the cell supernatants collected. Each samples was assayed in duplicate for TNFα using the L929 bioassay. The many preliminary experiments indicated that the cells *were* capable of producing large amounts of TNFα (i.e. in response to LPS).

Control and Removal of Endotoxin

All the media containing additional molecules that were to be incubated on the cells were first tested for the presence of endotoxin (LPS) using a limulus amebocyte lysate test (E-Toxate, Sigma). Those solutions testing positive for endotoxin were passed through an endotoxin binding column (Detoxi-Gel, Pierce) before use. This column consists of polymyxin B immobilized on agarose. Polymyxin B contains a cationic cyclopeptide with a fatty acid chain that can bind to and neutralize the lipid A portion of bacterial lipopolysaccharide (Issekutz, 1983: Morrison *et al.*, 1976). The gel was first washed with endotoxin free tissue culture water, and the sample then added and collected by gravity flow. The column was regenerated by washing with 5 bed volumes of 1% deoxycholate. The detergent was removed by washing again with pyrogen-free water. Samples were again tested for endotoxin and passed through the column repeatedly, until free of detectable endotoxin.

TABLE 8.1

Experimental Groups	Overnight Incubation Medium (4°C)	6 h Activation Medium (36°C)	(+)/(-) IFNγ
Part A: Controls			
Group 1	STD	STD	-
Group 2	whole-IgG	STD	-
Group 3	α-OA-IgG	STD	-
Group 4	STD	OA	-
Group 5	STD	α-IgG pAb	-
Group 6	STD	α-IgG mAb	-
Group 7	STD	IFNγ	+
Part B: IgG plus pAb		Dilution of pAb	
Group 8	whole-IgG	100	-
Group 9	whole-IgG	500	-
Group 10	whole-IgG	1000	-
Group 11	whole-IgG	2000	-
Group 12	whole-IgG	100	+
Group 13	whole-IgG	500	+
Group 14	whole-IgG	1000	+
Group 15	whole-IgG	2000	+
Part C: IgG plus mAb		Dilution of mAb	
Group 16	whole-IgG	100	-
Group 17	whole-IgG	500	-
Group 18	whole-IgG	1000	-
Group 19	whole-IgG	2000	-
Group 20	whole-IgG	100	+
Group 21	whole-IgG	500	+
Group 22	whole-IgG	1000	+
Group 23	whole-IgG	2000	+
Part D: α-OA IgG plus OA		Molar Ratio OA:IgG	
Group 24	α-OA-IgG	2.0	-
Group 25	α-OA-IgG	1.0	-
Group 26	α-OA-IgG	0.5	-
Group 27	α-OA-IgG	0.25	-
Group 28	α-OA-IgG	2.0	+
Group 29	α-OA-IgG	1.0	+
Group 30	α-OA-IgG	0.5	+
Group 31	α-OA-IgG	0.25	+

Summary of experimental groups for IgG activation of AM. Refer to text for description.

Detection of TNFα in Supernatants

As described in Chapter 5, page 92. All media containing added compounds (i.e. IgG, OA, etc.) were assessed for their effect on L929.8 cells; none were shown to have cytotoxic effects on the L929.8 cells. All supernatants were assayed in duplicate and on the same day. Each concentration point of the standard curves in each assay was done in triplicate.

Data Analyses

Data were analyzed using SigmaStat software. Differences between groups were compared by Student's "t"-test. Differences among groups were examined using ANOVA and Student-Newman-Keuls' tests. Data are expressed as average values ± SEM. Significance was assumed at the 5% level.

RESULTS

Calculation of the Extinction Coefficient for Guinea-Pig IgG

The absorbance at 280 nm of each sample was plotted against the concentration (0.1 to 0.8 mg/ml). The equation for the straight line was ($y = 1.44 x - 0.006$, $R^2 = 0.9996$) and the value for the slope (1.44) was taken to be the extinction coefficient.

Anti-ovalbumin Immunoglobulin Purification

The PCA results show that serum obtained from animals that were immunized as described in methods, does not contain detectable amounts of IgE antibody. This is in agreement with others whom report that sensitization with a large dose of antigen in the absence of an adjuvant induces preferential formation of IgG antibody (Benacerraff et al., 1968; Hicks & Okpaka, 1968; Ovary *et al.*, 1976). Sensitizing antibodies were

apparent when challenge was performed at 2 d, however these did not persist into 7 d. Also, the effect present at 2 d was not sensitive to heating (4 h at 56°C). The positive control serum for IgE gave positive results when animals were challenged at day 7; this response was sensitive to heat. Thus, our serum to be purified for use in our IgG-activation studies, contains no detectable IgE antibody, and does contain IgG antibody (i.e. short-term sensitizing antibody, detectable at 2 d).

From the T-Gel purification, the A_{280} of a 1:10 dilution of the combined and concentrated bound eluates was 0.517; the final volume of the sample was 157 ml. The amount of protein, using 1.44 as an extinction coefficient was therefore 10 x (0.517/1.44) = 3.590 mg/ml (563.67 mg of total protein). The results of the SDS-page electrophoresis indicate that the product is > 90% pure guinea-pig IgG.

From the OA affinity purification, the A_{280} of the combined and concentrated bound eluates (after dialysis) was 0.417. The final volume of the sample was 62 ml. The amount of protein, using 1.44 as an extinction coefficient was therefore (0.417/1.44) = 0.2896 mg/ml (17.95 mg of total protein). The SDS-Page electrophoresis showed light banding for the reduced IgG molecule with no other visible contaminants.

Binding Studies

A competitive binding plot for the binding of ^{125}I-IgG to guinea-pig AM is shown in Figure 8.1. The dissociation constant, Kd, was calculated according to the following formula (Akera & Cheng, 1977):

$$K_d = C_{0.5} - a$$

where: Kd is the dissociation constant; $C_{0.5}$ is the concentration of unlabelled IgG giving 50% inhibition of specific binding; a is the concentration of labelled IgG.

The data are from one experiment and the values represent the averages of three

observations for each concentration tested. The amount of ^{125}I-IgG specifically bound was calculated by subtracting the radioactivity bound in the presence of an excess of unlabelled IgG. Approximately 2% of the labeled protein bound non-specifically. This value was subtracted from the calculated bound amounts of protein. Half-maximum of specific binding was achieved at 1.24×10^{-9} Molar for ^{125}I-IgG.

The number of binding sites, B_{max}, was calculated using the following formula (Akera & Cheng, 1977):

$$B_{max} = B_a \frac{Kd + a}{a} = B_a \frac{C_{0.5}}{a}$$

where: $C_{0.5}$ is the concentration of unlabelled IgG giving 50% inhibition of specific binding; Ba is the concentration of labelled IgG bound to cells in the absence of unlabelled IgG; Kd is the dissociation constant; and a is the concentration of labelled IgG. This value was expressed in μg of protein. It was converted to moles and multiplied by Avagadro's number (6.022×10^{23}) to determine the number of molecules. This amount was then divided by the number of cells (5×10^5 cells/sample) to determine the number of molecules or binding sites per cell. The number of binding sites calculated was 1.54×10^5 sites per cell. The plot of the data was linear, suggesting that in these studies a single class of receptors for IgG monomer exists.

Activation Studies

Experimental results are shown in Figure 8.2. The data shown here are from one large experiment performed over one time period, and are consistent with the data from initial studies that evaluated and optimized conditions for individual groups. In this study, the cells obtained from the bronchoalveolar lavage fluid of eight guinea-pigs were combined. Macrophages were isolated by adherence, and four samples of each

experimental condition (i.e. each concentration of compound) were established. Each sample was then assayed for TNFα (in duplicate) using the L929 bioassay.

In *Part A*, the control groups show background secretion of TNFα. Standard media alone on cells resulted in background production of TNFα (2246 ± 248 pg/ml). Incubation of cells overnight with whole guinea-pig IgG then aspiration and incubation with standard media alone for 6 h resulted in TNFα production (3278 ± 403 pg/ml). Incubation of cells overnight with purified polyclonal anti-OA guinea-pig IgG then aspiration and incubation with standard media alone for 6 h resulted in TNFα production (2152 ± 218 pg/ml). Cells incubated overnight with standard media alone, then for 6 h with anti-guinea-pig IgG mAb (ascites fluid diluted 1:100 in standard medium), anti-guinea-pig polyclonal antiserum (diluted 1:100 in standard medium), OA solution (in standard medium at 2 times the molar concentration of IgG used), or with IFNγ (1.0 U/ml) produced 1289 (± 95), 1496 (± 309), 11600 (± 551), 1266 (± 75) pg/ml of TNFα, respectively.

Cells from *Part B*, the pAb test group, do not show enhanced TNFα secretion (i.e. above the sum of TNFα secretion observed for whole IgG and pAb antibody control groups). Incubation of cells with IFNγ however, significantly *decreased* background TNFα secretion in the first three dilutions of antibody evaluated ($p < 0.001$).

As well, the cells from *Part C*, the mAb test group, do not show enhanced TNFα secretion (i.e. above the sum of TNFα secretion observed for whole IgG and mAb control groups). Incubation with IFNγ did not significantly change these values.

Cells from *Part D*, produced large amounts of TNFα (range from 5846 ± 485 pg/ml to 10618 ± 685 pg/ml), however these are not significantly different from that observed from the OA solution control group alone. The production is concentration-

dependent, suggesting that OA alone can cause TNFα production from guinea-pig AM, possibly by a receptor-mediated process. Production due to endotoxin contamination is unlikely. At the highest concentration used, the OA solution tested negative for LPS in the limulus amebocyte lysate assay (Sigma). However, the sensitivity of the limulus assay is in the nanogram range therefore endotoxin contamination cannot be ruled out entirely. Interestingly, TNFα secretion was inhibited when cells in this group were incubated in the presence of IFNγ ($p < 0.001$).

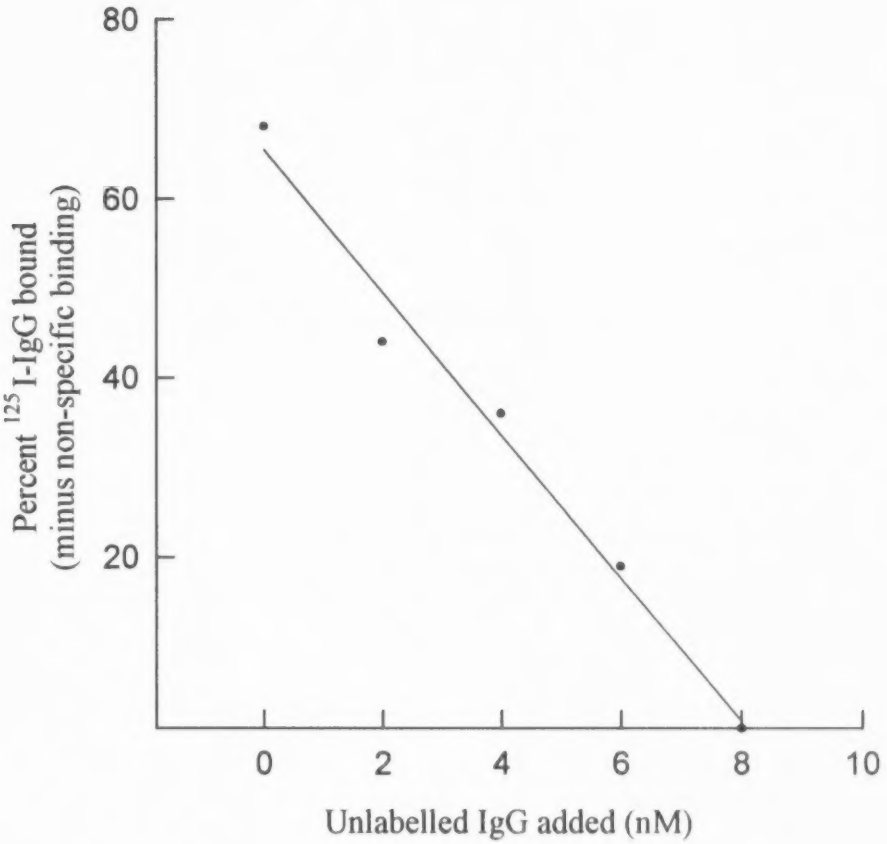

Figure 8.1. Competitive binding plot for the binding of radiolabelled guinea-pig IgG (^{125}I-IgG) to guinea-pig AM. AM were incubated with a constant amount (0.01 μg) of ^{125}I-IgG, and increasing amounts of unlabelled IgG. The concentration of unlabelled IgG is plotted against the percent of ^{125}I-IgG bound (with non-specific binding first subtracted). 50% inhibition of ^{125}I-IgG binding is achieved at 1.9 nM unlabelled IgG.

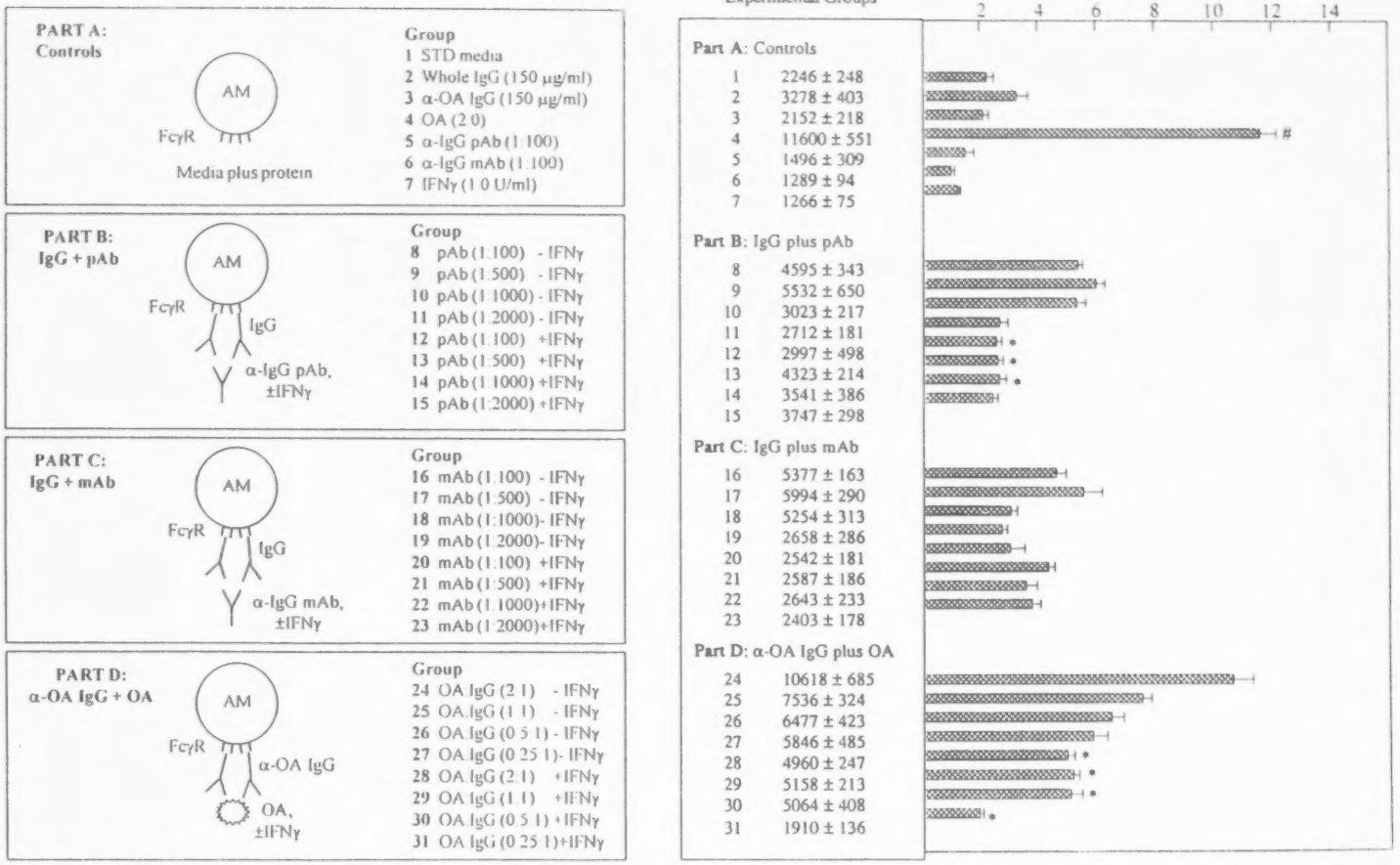

Figure 8.2 Results from IgG activation studies.

Legend for Figure 8.2. Experimental groups are indicated on the first page of the figure and correspond to those described in Table 8.1. On the second page of the figure, amounts of TNFα detected in each group are given (average ±SEM), and for ease of interpretation, are plotted to the right in a vertical bar plot.

Part A (groups 1 through 7) consists of *control groups.* All compounds used in experiments were incubated separately on AM and the supernatants tested for **TNFα.** **Group** 1 is incubation of cells with standard media; **Group 2** is incubation of cells with whole IgG; **Group 3** is incubation of cells with α- OA-IgG; **Group 4** is incubation of cells with OA solution; **Group 5** is incubation of cells with α-IgG pAb; **Group 6** is incubation of cells with α-IgG mAb; and **Group 7** is incubation of cells with IFNγ.

Part B (Groups 8 through 15) is the *anti-guinea-pig IgG pAb* test group. Cells were incubated in media that contained whole guinea-pig IgG for 30 min at 36°C, then overnight at 4°C. Media was aspirated and replaced with standard media containing anti-guinea-pig IgG polyclonal antiserum in various dilutions **(Group 8, 1:100, Group 9, 1:500, Group 10, 1:1000 and Group 11, 1:2000)** Cells were incubated for 6 h at 36°C. Supernatants were collected and assayed for **TNFα.** Groups **9, 10, 11** and **12** are the above dilutions of pAb, but prepared also with IFNγ (1.0 U/ml).

Part C (Groups 16 through 23) is the *anti-guinea-pig IgG mAb* test group. This part of the experiment is identical to that described in Part B, however mAb was used instead of pAb. (**Groups 16 through 19** are without IFNγ; **Groups 20 through 23** are with IFNγ).

Part D (Groups 24 through 31) is the purified *anti-OA guinea-pig polyclonal IgG antibody (α-OA-pAb) plus antigen (OA)* test group. Cells were incubated in media that contained α-OA-pAb for 30 min at 36°C, then overnight at 4°C. Media was aspirated and replaced with standard media containing OA in various molar dilutions of OA to α-OA-pAb (**Group 24**, 2:1, **Group 25**,1:1, **Group 26**, 0.5:1, and **Group 27**, 0.25:1). **Groups 28, 29, 30** and **31** are the above dilutions of OA, but prepared also with IFNγ. Cells were incubated for 6 h at 36°C. Supernatants were collected and assayed for TNFα.

# Denotes significantly different from all other control groups. We compared the effect of IFNγ *within* each experimental condition. * Denotes significantly different in the presence of IFNγ.

DISCUSSION

Current evidence suggests that the early asthmatic reaction is predominantly mast cell mediated. Mucosal mast cells are induced to secrete a variety of inflammatory and spasmogenic mediators upon perturbation of high affinity cell surface IgE receptors (FcεRI), by allergen or other stimuli (Lai *et al.*, 1988; Howarth *et al.*, 1987). Mast cells from symptomatic atopic and nonatopic asthmatics have increased immunoreactivity for TNFα, IL-3, IL-5, and GM-CSF (Ackerman *et al.*, 1994). In this same study, macrophages and monocytes were also a source of increased IL-1, GM-CSF and TNFα immunoreactivity. AM obtained from the BAL of patients suffering from sarcoidosis, pulmonary fibrosis, and acute respiratory distress syndrome produce significant amounts of IL-1β, TNFα, IL-6 and IL-8 compared to controls (Steffen *et al.*, 1993; Tran Van Nhieu, 1993). As well TNFα mRNA-positive cells were increased in frequency in the BAL fluid from asthmatics compared to nonatopic control patients (Ying *et al.*, 1991).

Studies in our laboratory suggest that AM are activated by antigen specific IgG plus antigen to secrete TNFα. Using a guinea-pig model of asthma, naive guinea-pigs were passively immunized with antigen specific (OA) serum that was demonstrated to be of an IgG-class. Animals then challenged by aerosolized antigen (OA) had detectable levels of TNFα in their bronchial alveolar lavage fluid from between 30-60 min post challenge. Amounts of TNFα increased as the time interval between challenge and lavage was increased (Kelly *et al.*, 1992). AM retrieved from the lavage fluid were also upregulated to secrete TNFα (Denis *et al.*, 1994). Receptor cross-linkage mechanisms may account for the activation of cells by immunoglobulins (Ig). Receptors can be cross-linked by receptor-bound Ig that is exposed to the appropriate antigen (i.e. Type I allergic processes analogous to the activation of mast cells via Fcε receptors), or by preformed immune complexes such as Ig plus antigen or Ig aggregates. Most literature

describes mechanisms of soluble- and immobilized-immune-complex activation of monocytes and macrophages, with regard to the secretion of various mediators. Activation of Fcγ receptors by bound monomeric IgG, followed by antigen has not been thoroughly investigated in the context of asthma.

Recent studies have described the capacity of antigen (OA) to induce histamine and leukotriene (LTB_4 and LTD_4)l release by lung tissue extracted from guinea-pigs that were passively sensitized *in vivo*, with purified antigen specific (OA) IgG1 antibody (Cheng *et al.*, 1990). Also, anaphylactic histamine release has been described from lung and tracheal tissues extracted from actively sensitized guinea-pigs (sensitized to respond with IgG antibodies) then challenged *in vitro* with antigen (Van Amsterdam *et al.*, 1991). Further, guinea-pig mast cells and AM can be passively sensitized, *in vitro*, with antigen (OA) specific IgG1 or IgG2 antibodies, and then activated to secrete histamine (by mast cells) and TXA_2 (by macrophages) upon OA challenge (Al-Laith *et al.*, 1993). These studies demonstrate the involvement of antigen specific IgG, antigen and AM in Type I allergic responses in the airways of guinea-pigs.

Studies from the laboratory of Koyama and colleagues have focused on characterizing Fcγ interactions on guinea-pig peritoneal macrophages. Using immune complexes of OA plus purified OA-specific guinea-pig IgG1 or IgG2, they have demonstrated isotype specific rises in O_2- generation, intracellular calcium concentrations, phosphatidylinositol turnover and intracellular arachidonic acid release (Imamichi *et al.*, 1990; Imamichi and Koyama, 1991(a); Imamichi and Koyama 1991(b)). In similar studies, secretion levels of these mediators by guinea-pig AM were also increased in response to binding of murine monoclonal antibodies directed against two known isoforms of the Fcγ receptor followed by cross-linking of the receptors by anti-murine IgG $F(ab')_2$ (Shimamura *et al.*, 1987). Thus, in these studies, FcγR cross-

linkage on guinea-pig AM, is sufficient to generate various second messenger molecules.

In this study, we first determined the association constant for the binding of whole monomeric IgG to FcγR on guinea-pig AM. Half-maximum of specific binding (K_a) was achieved at 1.24×10^9 M^{-1} for ^{125}I-IgG. Problems arise in trying to assess FcγR affinities for IgG. Many investigators have used immune complexes to detect and report the presence of cell surface Fcγ receptors as well as receptor affinity for IgG. Because of the heterogenous nature of immune complexes, quantitative estimates of binding affinities should not be made, nor do such experiments reflect the binding of IgG alone. Binding of monomeric IgG and immune complexes differ in that various receptor isotypes exist with different binding affinities for monomeric or complexed IgG. Three classes of receptors for the Fc region of IgG have been identified in mice and humans. Each show different binding affinities for IgG, depending on whether IgG is in a complexed or monomeric form (Anderson & Loony, 1987). For guinea-pig Fcγ receptors, the literature is limited. Association constants for the binding of guinea-pig IgG immune complexes (OA plus anti-OA-IgG2 antibody) to guinea-pig peritoneal macrophages is reported to be approximately 1.7×10^8 M^{-1} (Nakamura *et al.*, 1988). The association constant for binding of monomeric guinea-pig IgG1 and IgG2 to guinea-pig peritoneal macrophages is recorded as 2.45 ± 1.08 10^6 M^{-1}, and 3.39 ± 0.61 10^6 M^{-1}, respectively (Gorczyca *et al.*, 1992). Also, it has been shown that as well as homologous IgG2, rabbit IgG interacts with guinea-pig peritoneal macrophages (Janusz *et al.*, 1983; Janusz *et al.*, 1980), and binding affinities for this protein to the Fcγ receptor are reported as 5.27 ± 0.17 10^7 M^{-1} (Gorczyca *et al.*, 1989). The number of AM Fcγ binding sites determined in our study was 1.54×10^5 sites per cell. In accordance, Gorczyea *et al.* (1992) report the number of binding sites for guinea-pig IgG1 to guinea-pig peritoneal macrophages as $2.62 \pm 1.33 \times 10^5$, and for IgG2 as $5.59 \pm 0.82 \times 10^5$ sites per cell. Using guinea-pig peritoneal macrophages, Leslie & Cohen

(1976) report values approximately a log fold higher. Thus, in our studies, monomeric whole IgG binds to a high affinity receptor on guinea-pig AM.

Further, we investigated whether AM could secrete TNFα as a consequence of FcγR cross-linkage. Experiments were designed to evaluate the hypothesis that, analogous to IgE on mast cells, cross-linkage of monomeric bound IgG (and thus cross-linkage of Fcγ receptors) can activate AM to produce TNFα. Monomeric whole guinea-pig IgG was first bound to guinea-pig AM. Cells were then incubated in the presence of rabbit anti-guinea-pig IgG polyclonal antiserum or murine anti-guinea-pig IgG monoclonal antibody. A parallel experiment evaluated binding of antigen specific IgG, followed by incubation with antigen. Results are shown in Figure 8.2; in all experimental conditions, guinea-pig AM did not produce amounts of TNFα above that observed from control groups, however these results should be interpreted cautiously in the absence of a good positive control (such as LPS activation of cells to produce TNFα). Thus, unlike immediate hypersensitivity reactions encountered with mast cells and IgE, guinea-pig AM were not activated to secrete TNFα via cross-linkage of IgG (bound on their cell surface) by soluble α-IgG-antibody or antigen.

Debets *et al.* (1988) showed that when human monocyte Fc receptors were cross-linked by soluble or insoluble immune complexes, solid phase antibody, or antibody-coated phagocytizable particles, they secreted large amounts of TNFα; this secretion was rapid, particularly in the case of soluble immune complexes. On the basis that murine IgG2a binds to human FcγRI and murine IgG1 binds to human FcγRII, further studies by these investigators were undertaken to assess receptor cross-linking. On freshly isolated, untreated human monocytes, cross-linking of FcγRI with solid phase murine IgG2a induced TNFα secretion. Cross-linking with solid phase murine IgG1 did not induce TNFα secretion from FcγRII. In this study, it was not possible to induce TNFα

secretion by cross-linking FcγRI or FcγRII with anti-FcγR mAb. Nor was it possible to induce TNFα production with soluble or solid-phase anti-murine IgG when murine IgG was bound to the cells (Debets *et al.*, 1990). In these studies, TNFα was produced only as a consequence of immobilized or soluble IgG *immune complexes.* In our study, cross-linking of monomeric IgG by soluble antibody or antigen was not sufficient to generate TNFα production. These observations are suggestive of the requirement of enhanced antibody-antigen avidity for receptor interactions to take place. Avidity is a measure of the overall stability of the complex between antibodies and antigens. Antibodies that bind to multiple sites on an antigen (as in immune complex formation) can form large, stable, multimeric complexes. When an antigen is coated by many antibodies, the multimeric complex provides multiple binding sites in a flexible geometric arrangement that allows stable multimeric interactions with secondary agents, such as receptors. In this same way, antigens (or antibodies) immobilized on solid supports (usually in high concentrations) increase the avidity of substrate (i.e. receptor) binding. This arrangement for binding may facilitate interactions with other cell surface molecules necessary for generating some cellular signals.

The ability of macrophages to produce TNF is modulated by exposure to several different cytokines, IFNγ being one that is well studied. In various experimental systems, IFNγ was shown either to directly induce TNFα production (Urban *et al.*, 1986; Philip & Epstein, 1986) or to prime macrophage for TNF production in response to LPS or other stimuli (see review by Trinchierri, 1991). We tested the ability of IFNγ to prime cells for TNFα secretion after incubation with IgG (whole or antigen specific) then activation with anti-IgG antibody or antigen (for 6 h). Interestingly, IFNγ had an *inhibitory* effect on TNFα production induced by whole IgG plus anti-IgG mAb and also with antigen specific IgG plus antigen. Similar results were not obtained in the pAb experimental group. The reasons for such inhibition are unclear. In priming cells for

tumor cytotoxicity with IFNγ, cytotoxicity occurs as the final step of a reaction sequence requiring various trigger signals (Meltzer, 1981). In our study, these "priming" conditions may still need to be optimized. Alternatively, it is possible that IFNγ activates the macrophages to secrete proteolytic enzymes which would degrade the secreted TNFα. The biological activity of TNF as detected in the L929 bioassay would thus be diminished. Also, IFNγ has been described as one of the most species-specific cytokines and has the most restricted host range of activity of the interferons (Terrell & Green, 1993). No studies presently exist specifically evaluating the activation of guinea-pig AM by murine IFNγ, however recombinant human IFNγ has been shown to have biological effects on guinea-pig airway strips, *in vitro* (Chen *et al.*, 1994). The biological responses of guinea-pig AM to murine IFNγ may require further characterization.

An interesting observation from this study that warrants further investigation is the observation that OA causes TNFα secretion from naive guinea-pig AM. This effect seems *not* to be attributable to an interaction between anti-OA IgG plus antigen as OA alone caused similar amounts of TNFα production as that caused by antibody plus OA. All solutions used in experiment tested negative for endotoxin (limulus amebocyte lysate assay, sensitivity of approximately 10 to 25 pg/ml) however, endotoxin contamination cannot be entirely ruled out by any known procedure. Further experiments done in the presence of polymyxin B (an inactivator of endotoxin) would provide useful information. The OA molecule is a mannose terminated glycoprotein, and would therefore be expected to bind to mannose (mannosyl-fucosyl) receptors. Glycosylated proteins such as enzymatically inactive horseradish peroxidase and mannosylated bovine serum albumin have been shown to induce TNFα secretion from murine peritoneal macrophages, likely through a mannose receptor (Lefkowitz *et al.*, 1991). Recently it has been proposed that mannose receptor activation can cause TNFα secretion from

human monocytes (Polat *et al.*, 1993). Such a receptor-mediated response would exhibit dose-dependency such as that demonstrated in this study.

In normal lungs AM are immune modulators and serve as the first line of defense to inhaled particulates. They phagocytose and degrade particulate matter, keeping the inflammatory response regulated and contained, thus providing a safeguard against excessive immunologic damage by inhaled material (Djukanovic *et al.*, 1990; Herscowitz, 1985; Holt, 1986). Macrophages isolated from human bronchial alveolar lavage fluid are a heterologous population and can be isolated phenotypically and functionally into subsets (Spiteri *et al.*, 1992; Poulter *et al.*, 1986). Using monoclonal antibodies, lung macrophages have been classified as phagocytes, antigen presenting cells or suppressor cells (Poulter *et al.*, 1986). Studies are now beginning to emerge that suggest that macrophages retrieved from asthmatic patients have a decreased suppressor function. The aberrant activation of macrophages, their phenotypical change and thus loss of regulatory function may account for their involvement in inflammatory diseases such as asthma. Macrophages used in our study were from naive animals. It would be useful to repeat our experiments using guinea-pig AM from animals that first received a course of passive immunization and aerosoled antigen challenge (*in vivo*). Perhaps a particular stage of macrophage activation is necessary to demonstrate the activation mechanisms under investigation.

CHAPTER 9

SUMMARY AND CONCLUSIONS

We hypothesized that TNFα is an important early mediator contributing to airways' hyperreactivity and inflammation. We began our studies using a guinea-pig model of anaphylaxis in which the animals were passively immunized with anti OA containing serum and then challenged with OA antigen by inhalation. The first set of experiments were designed to detect *early* antigen-driven production of TNFα and thus we evaluated the levels of TNFα in the BAL fluid at various times post antigen challenge. We demonstrated that TNFα production was upregulated in this guinea-pig model of asthma (Chapter 2). TNFα was present in BAL fluid from between 30 and 60 min after antigen challenge, and increased in amounts with time at 90 and 120 min post challenge. The peak of this secretion was not defined and further time periods are worth characterizing. TNFα was not detected in the lavage fluid from unchallenged animals, whereas animals that inhaled LPS (as a positive control) had amounts of TNFα in BAL fluid significantly larger than the antigen challenged group, at all time periods examined. LPS-induced TNFα secretion was highest at 90 min post challenge and tapered at 120 min, thus suggesting different mechanisms of TNFα induction or regulation by the two stimuli (antigen or bacterial polysaccharide).

Using cell-based assays, we were unable to detect IL-1 or IL-6 in the lavage fluid, from these experiments. A factor present in the lavage fluid was cytotoxic to both the D10.G4.1 and B9 hybridoma cells lines used for IL-1 and IL-6 detection, respectively. It is worth noting that in order to preserve the bioactivity of TNFα (and thus its detection using the L929 bioassay) a protease inhibitor (aprotinin) was necessary in the lavaging solution.

Of further interest from these experiments is the suggestion that the antigen driven response is likely a consequence of IgG antibody. Previous PCA and ELISA work in our laboratory has shown that the serum used for passive immunization is void of detectable IgE and contains large amounts of antigen specific IgG antibody. Thus, we further hypothesized that stimulation of the cell surface receptors that bind IgG (Fcγ receptors) may be a likely mechanism inducing TNFα production (to be discussed later).

We next hypothesized that an important cell type contributing to the increased production of TNFα was the AM. As described in Chapter 3, AM were harvested from the BAL fluid from passively immunized then challenged guinea-pigs and incubated with or without zymosan, for a total of 18 h. Supernatants were then assayed for levels of TNFα, IL-1 and IL-6. Spontaneous production of TNFα and IL-6 was evident from cells collected at 30, 60, 90 and 120 min post antigen challenge. The production of TNFα increased as the time interval between challenge and lavage increased, and the maximal production was not defined. In contrast, AM collected from unchallenged animals did not spontaneously produce TNFα at any time periods examined. The production of IL-6 was similar at all time periods examined and significantly larger than the "background" production of IL-6 in the unchallenged groups. Spontaneous secretion of IL-1, was detected in similar amounts from the challenged and unchallenged groups. Thus, in our experiments, AM are driven by inhaled antigen to produce TNFα and IL-6.

By collecting cell supernatants at an arbitrary time (18 h), it is not clear when, or if, the cells have ceased to produce TNFα. What is worth noting however, is both the early (i.e. into lavage fluid) and continued (i.e. from cultured AM) production of this cytokine as a consequence of antigen challenge. This suggests a role for this cytokine and for AM, both in early and late hypersensitivity reactions in the lung.

As detailed in previous chapters, TNFα may have several roles in contributing to

the pathophysiology of allergic asthma. Importantly, TNFα-induces the expression of ICAM-1 on vascular endothelial cells and bronchial epithelial cells, and perfusion of monoclonal antibodies reactive to ICAM-1 results in the decrease of both bronchial hyperreactivity and eosinophil infiltration (Wegner *et al.*, 1990). Apart from the participation of IL-6 in inducing differentiation of B-lymphocytes and the production of IgE antibody (Vercelli *et al.*, 1988), the role for IL-6 in asthma is unclear.

The effect of zymosan on cytokine secretion, is noteworthy. Zymosan-induced TNFα production equaled and at some time points, exceeded that observed in the zymosan-stimulated LPS positive control group. Thus, AM from antigen challenged animals are maximally primed to secrete large amounts of TNFα subsequent to further stimulation. Zymosan also enhanced IL-6 production, but not to the levels observed in the zymosan-stimulated LPS control groups. IL-1 secretion was enhanced by zymosan stimulation and IL-1 levels became equivalent to zymosan-stimulated LPS groups, however production did not exceed that from negative control groups (i.e. unchallenged groups).

The fact that, in our model, antigen challenge *primes* macrophages for production of large amounts of TNFα subsequent to a second stimulus, is important. Cytokine production has been studied from AM collected from allergic patients 18 h after a bronchial allergen challenge (Gosset *et al.*, 1992a). The results demonstrate a significant increase in TNFα and IL-6 in AM supernatants from patients exhibiting a late response, in comparison with that of patients developing only an early asthmatic reaction or no response at all. Thus, it appears that the presence of TNFα is necessary for both initial and later events in the complex processes that lead to inflammatory responses in the lungs.

The next set of experiments examined the effects of capsaicin pretreatment of The

guinea-pigs, on TNFα production following antigen challenge in passively immunized animals (Chapter 4). The hypothesis was that tachykinin neuropeptides may partly regulate TNFα production in our *in vivo* model, and also from AM, *in vitro*. Lavage was performed at 90 min post challenge based on the previous experiments demonstrating a significant production of TNFα into lavage fluid and also from cultured AM at this time point.

Elimination of neuropeptides from sensory fibers had no effect upon the amounts of TNFα appearing in the lavage fluid after antigen challenge or LPS inhalation. Thus, tachykinins such as SP and NKA appear *not* to be involved in the immediate production of TNFα following antigen challenge. Also, further *in vitro* experiments performed, investigating the ability of potent NK1 and NK2 receptor agonists ([Sar, $Met(O_2)^{11}$]-substance P, and [β-Ala^8]-neurokinin-A 4-10, respectively, (purchased from Penninsula Laboratories)) to directly induce TNFα production, from guinea-pig supports this conclusion. Incubation of naive guinea-pig AM with these potent and selective NK1 and NK2 receptor agonists (ranging in dose from 10^{-5} to 10^{-10} Molar), for 3 h, did not induce TNFα production from these cells (data not shown). However, the spontaneous and zymosan-stimulated production of TNFα from cultured AM from capsaicinized/ challenged guinea-pigs was suppressed compared to non-capsaicinized/challenged animals.

Thus, it appears that there is a time course for neuropeptide involvement in TNFα production by antigen activated AM. After an initial production of TNFα by AM (i.e. post antigen challenge), neuropeptides may offer the second signal to subsequent TNFα production from these antigen-primed cells. Also, the presence of neuropeptides may well exceed their observed effects on macrophage TNFα production, and such time courses are worthy of further investigation.

Chapter 5 details the development of our cell-culture system to further investigate, mechanisms of TNFα production by guinea-pig AM, *in vitro*. Macrophages are commonly isolated from samples (blood and lavage fluid) by their selective adherence to plastic, when other cells do not. An important finding of this study was that guinea-pig AM are activated to secrete low levels of TNFα as a consequence of their isolation by adherence to plastic. Thus, it is important to bear in mind the phenotypical changes that can occur with cells as they are handled *in vitro*.

In this study we also demonstrated, by discarding the first 10 ml of lavaged fluid from each animal, many of the contaminating cell types (primarily eosinophils) are decreased in numbers, thus providing a means for "enriching" for AM. Percoll gradient separations were also attempted as a means to purify AM from lavage fluids, however this was not as successful. The introduction of further manipulations with the cells made it more difficult to control for endotoxin. As well, many cells are lost during Percoll separation, rendering it inefficient in terms of the number of animals that were then required for lavage. Thus, in the following *in vitro* studies investigating thalidomide's ability to inhibit LPS-induced TNFα secretion, AM were collected and enriched for by discarding the first 10 ml of lavaged fluid, and then cultured under non-adherent conditions.

In the next set of experiments (Chapter 6) we tested the hypothesis that thalidomide, a known inhibitor of TNFα, may alter airways' hyperreactivity. Thalidomide has been described as an immunomodulating agent, and has been shown to selectively decrease the stability of TNFα-mRNA (Moreira *et al.*, 1993). First, we performed *in vitro* studies to demonstrate thalidomide inhibition of LPS-induced TNFα secretion from guinea-pig AM. We examined the racemic mixture as well as both diastereomers of thalidomide, and showed that the (-) diastereomer is more potent than

the (+) diastereomer at inhibiting TNFα production. Experiments done *in vivo* then evaluated the ability of this compound to alter histamine- or LTC_4-induced bronchoconstriction in our antigen-based model of airway hyperreactivity (i.e. that of passive immunization of guinea-pigs followed by aerosoled antigen challenge). Thalidomide (infused over 3 days by osmotic pump) inhibited the increase in airway resistance induced by histamine in our model of antigen-elicited airway hyperreactivity. When LTC_4 was the bronchoconstricting agonist, airway hyperreactivity was difficult to demonstrate, thus thalidomide had nondefinitive effects. We cannot conclude that the inhibitory effects of thalidomide on histamine-induced bronchoconstriction (post antigen-induced airway hyperreactivity) are a consequense of its demonstrated ability to inhibit TNFα production (*in vitro*). However, such an effect is likely due to the many immune-modulating properties of this compound.

The last set of experiments addressed the hypothesis that allergen activation of AM to produce TNFα may occur via cross-linking of their cell surface Fcγ receptors (Chapter 8). First, we attempted to develop a cell line (heterohybridoma) secreting an anti-OA antibody of a guinea-pig IgG class, for use in *in vitro* AM activation studies (Chapter 7). This, however, proved to be unsuccessful, likely on account of the genetic instability of the fused product from two different species. This is a common hindrance to successful inter-species hybrids. The desired antibody polyclonal was hence purified from the serum of actively immunized guinea-pigs.

In our final experiments (Chapter 8), we used ^{125}I-radiolabelled whole guinea-pig IgG to define an association constant for the Fcγ receptor on guinea-pig AM that binds monomeric IgG. The association constant was determined to be $1.24 \times 10^9\ M^{-1}$, and the number of binding sites per cell was estimated to be 1.54×10^5. These values are consistent with those in the literature and demonstrate within our system, the

detection of a high affinity Fcγ receptor that binds monomeric IgG.

Next we investigated whether the activation of AM by allergen to secrete TNFα involved the cross-linking of Fcγ receptors via cell-bound IgG. Cells were incubated with whole guinea-pig IgG (bought commercially), or anti-OA IgG (purified from serum of actively immunized animals). The bound whole IgG was then cross-linked by the addition of anti-guinea-pig IgG monoclonal or polyclonal antibody. The bound anti-OA IgG was cross-linked by the addition of OA antigen. All three if these experimental conditions failed to induce production of TNFα above the background levels produced by control groups. In the absence of a good positive control however, these results need to be interpreted cautiously. It appears though, that in these experiments, IgG-induced TNFα production from guinea-pig AM does not occur through mechanisms that mimics the activation of mast cells by IgE (i.e. receptor cross-linkage via monomeric bound immunoglobulin plus inhaled antigen). Within the lungs, it is possible that immune complexes of antibody and inhaled antigen form, bind to Fcγ receptors and elicit TNFα production. Immune complexes and immobilized IgG are known to activate cells to secret TNFα (detailed in Chapter 8). *In vitro* experiments addressing this mechanism of cellular activation would provide useful information.

The role of IgG and Fcγ receptors in allergy, is worthy of full investigation. The structure of the high affinity FcεRI is now known to be a tetrameric complex, composed of one α chain, one β chain, and a dimer of identical disulfide-linked γ chains (Kinet, 1990). The γ-chains of the FcεRI each contain 18 member amino acid signalling motifs on the cytoplasmic c-terminal tails (Kinet, 1992). These same γ-chains exist as homodimers or heterodimers in the human FcγRI and RIII receptors, respectively (van de Winkel & Capel, 1993) and are essential for cell surface receptor expression and signal transduction. It has also been shown that in mast cells, in addition to the dimeric

γ-chains, the same β-subunit is associated with FcεRI and the FcγRIII (Kurosaki *et al.*, 1992). These authors show that the two receptors differ only in their ligand recognition subunits, and that through the association of alternative ligand recognition subunits (α_ε, α_γ), a common signal transduction complex ($\beta\gamma_2$) mediates similar biochemical and effector functions in response to IgG and IgE. To add further to the evidence for such structural and functional Fc receptor homogeneity, is the discovery that the low affinity IgE receptors on mouse mast cells and macrophages, appear to be FcγRII and FcγRIII receptors (Takizawa *et al.*, 1992). Thus, the so-called receptors for IgG (FcγRII and FcγRIII) are not isotype specific; they bind IgE and are thus functional Fcε receptors as well as Fcγ receptors. It is possible therefore, that in conditions of excess IgE production (i.e. atopy), IgG receptors may be activated by immunoglobulin E, to secrete various biochemical mediators. Further complicating these poorly understood mechanisms, is the demonstration that murine low affinity IgG receptors can regulate high-affinity IgE receptor-mediated mast cell release of mediator (serotonin) and cytokine (TNFα) (Daeron *et al.*, 1995). This inhibitory effect requires cross-linkage of the two receptors by the same multivalent ligand and is reversible upon disengagement.

Apart from these structural studies, a recent manuscript describes antigen-specific IgG1 and IgG3 degranulation of human eosinophils, collected from short ragweed pollen sensitive patients with asthma (Kaneko *et al.*, 1995). Thus, there is increasing evidence for structural and functional homogeneity of Fcε and Fcγ receptors. This infers the likely involvement of Fcγ receptors (like Fcε receptors) in allergy. Receptor-mediated events following allergen exposure are extremely complex, and on the molecular level may involve interactions between Fcε and Fcγ receptors. It could be postulated, that allergic disease may in part be a consequence of aberrations in the structure and or function of components of Fcγ receptors. Possible roles for IgG and Fcγ receptors in allergy are only now emerging and require much further elucidation.

An interesting finding from these activation studies, was the observation that the OA molecule can induce TNFα secretion on its own. This may be through the stimulation of mannose receptors that are known to bind OA.

In conclusion, these studies have demonstrated the early release of TNFα into BAL fluid of guinea-pigs, as a consequence of antigen challenge. Also, AM collected post challenge, secrete larger amounts of TNFα and IL-6 (both spontaneously and in response to zymosan) than those collected from unchallenged animals. This may be indirectly modulated by the tachykinin neuropeptides SP and NKA. Direct incubation of AM with SP and NKA analogs did not induce TNFα production, however, AM retrieved from capsaicinized animals had a diminished ability to produce TNFα (both spontaneously and in response to zymosan). Thalidomide inhibits LPS-induced TNFα secretion, *in vitro*. When used *in vivo*, thalidomide inhibits the increase in airway hyperreactivity to histamine in our antigen-based model of airway hyperreactivity. This is likely due to the immune-modulating effects of thalidomide. Whether or not the antigen-mediated increase in TNFα production demonstrated in our studies is mediated through IgG stimulation of Fcγ receptors on macrophages, remains to be determined. Experiments in Chapter 8 demonstrated that monomeric bound IgG plus anti-IgG antibody or antigen did not enhance TNFα production (i.e via stimulation of FcγR) from AM collected from naive guinea-pigs. Other mechanisms of FcγR activation are worthy of investigation; experiments using human AM derived from asthmatic and non-asthmatic subjects could provide relevant information.

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